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(54) Title: THERAPEUTIC COMPOSITIONS

(57) Abstract: Therapeutic siRNAs and methods of making and using are enclosed.

THERAPEUTIC COMPOSITIONS

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/415,698, filed October 2, 2002.

BACKGROUND

RNA interference or "RNAi" is a term initially coined by Fire and co-workers to describe the observation that double-stranded RNA (dsRNA) can block gene expression when it is introduced into worms (Fire *et al.* (1998) *Nature* 391, 806-811). Short dsRNA directs gene-specific, transcriptional and post-transcriptional silencing in many organisms, including vertebrates, and has provided a new tool for studying gene function.

SUMMARY

The present invention relates to a method of modulating, preferably decreasing, gene expression.

The method includes: administering a first RNA that is substantially identical to a region of the target gene, or a DNA which encodes the first RNA; administering a second RNA that is substantially identical to a region of the target gene, or a DNA that encodes the second RNA; thereby modulating gene expression. In a preferred embodiment the method modulates, and preferably decreases, transcription.

Thus, the method includes the use of multiple RNAs directed to the target gene, where preferably the RNAs are different from one another. RNAs or nucleic acids are different if they have a sequence that differs by at least one nucleotide. Thus, by way of example, two RNAs that are substantially identical to two different regions of a target gene are different. By way of example, different RNAs, or generally nucleic acids, can differ by 1, or more nucleotides. By way of example, a nucleic acid which extends in one direction one or more bases is different from a nucleic acid that lacks that one or more bases. By way of example, different nucleic

acids can have regions of overlap or can be entirely distinct. By multiple mRNA is meant two or more. In preferred embodiments at least 3, 4, 5, 10, 15, or 20 different RNAs are administered. In a preferred embodiment a sufficient number of different RNAs are administered such that one or more of the following is seen: there is a decrease in transcription of the target gene; there is an alteration in chromatin structure, preferably in the target gene; there is an alteration in chromatin structure, preferably in the target gene, which affects nucleic acid of more than one nucleosome or more than about 160 base pairs.

In a preferred embodiment at least one, and preferably more than one, of the RNAs is targeted to a region other than a coding region of the target gene. Exemplary noncoding regions include: A nontranscribed region, *e.g.*, a control region, *e.g.*, a 3' or 5' or unlinked control region, *e.g.*, a promoter, enhancer, or insulator. Other exemplary noncoding regions can include a transcribed noncoding region, *e.g.*, an intron, a 5' untranslated sequence (UTS) or a 3' UTS. Such regions may be absent from a precursor or mature mRNA.

In other embodiments at least two RNAs are targeted to a coding region.

In some embodiments at least one RNA is targeted to a noncoding region and at least one RNA is targeted to a coding region.

In a particularly preferred embodiment the method includes: administering a first RNA which is substantially identical to a noncoding region, *e.g.*, a nontranscribed region, *e.g.*, a 3' or 5' or unlinked control region, *e.g.*, promoter, enhancer, or insulator, sequence of a target gene, or a transcribed non coding region, *e.g.*, an intron, or a DNA which encodes the first RNA; administering a second RNA that is substantially identical to a region of the target gene, or a DNA that encodes the second RNA; thereby modulating gene expression. In a preferred embodiment the method modulates, and preferably decreases, transcription.

In a preferred embodiment, DNAs encoding the RNAs are administered. The DNA encoding the first and second RNAs can be on the same or different molecules.

In a particularly preferred embodiment the second RNA is substantially identical to a noncoding, *e.g.*, a nontranscribed region, *e.g.*, a 3' or 5' or unlinked control region, *e.g.*, promoter, enhancer, or insulator sequence of the target gene, or a transcribed noncoding region, *e.g.*, an intron, and differs in sequence by at least one

nucleotide from the first RNA. Preferably multiple RNAs, *e.g.*, at least 3, 4, 5, 10, 15 or 20, all targeted to a non coding *e.g.*, a nontranscribed region, *e.g.*, a 3' or 5' control region, or a transcribed non coding region, *e.g.*, an intron, are administered.

In a preferred embodiment the second RNA is substantially identical to a transcribed region, and differs in sequence by at least one nucleotide from the first RNA.

While not wishing to be bound by theory, an RNA, and in particular one which is targeted to a noncoding, *e.g.*, non transcribed region of a target gene, may act by causing a covalent modification of the target nucleic acid or a protein, *e.g.*, a histone associated with the target gene, which leads to a modulation, preferably a decrease, in transcription.

In a preferred embodiment, the RNAs result in one, and preferably more than one covalent modifications, preferably methylation, of the target gene or an associated protein, *e.g.*, a histone. In a preferred embodiment the modification is cleavage of the DNA, and can result in the excision of all or part of the targeted gene.

In a preferred embodiment the RNAs cause an alteration in the ability of the target gene to be transcribed. While not being bound by theory, an alteration in transcribability can be due to one or more covalent modifications, preferably methylation, of the target gene or an associated protein, *e.g.*, a histone. In particular, some embodiments may have a sufficient number of modifications, *e.g.*, at least 10, 50, 100, or 5,000, within less than 10, 100, 1,000, 10,000, or 100,000] nucleotides of the target, or in close proximity, or on an associated protein, *e.g.*, a histone, which may cause an alteration in chromatin structure.

In a preferred embodiment the modification is methylation. In other embodiments the modification can include: a change in the presence or absence or structure of a sugar molecule, acetyl group, ADP-ribose moiety, phosphate group, ubiquitin moiety, or sulfate group, as well as cleavage of a protein.

In a preferred embodiment the RNA has been modified to alter its distribution or lifetime, *e.g.*, the RNA has been modified to enhance nuclear localization. This can be done by inclusion of one or more phosphorothioate groups or by inclusion of an RNA nuclear retention signal, *e.g.*, all or an active part of a U6 snRNA retention

signal. This is particularly preferred for RNAs that are targeted to non-transcribed regions.

The use of multiple RNAs can provide a desired level of gene silencing with fewer administrations or by providing smaller amounts of dsRNA. In some embodiments multiple administration will decrease the frequency of administration by at least 1, and preferably 2, 3, or 5 administrations in a 6 or 12 month period, as compared to use of a single dsRNA. In some embodiments multiple administration will decrease the total amount of dsRNA, in terms of moles, or in terms of weight, by at least 10, 20, 30, or 50%, as compared to use of a single dsRNA. In some embodiments multiple administration, particularly provision of multiple RNAs targeted to noncoding, particularly nontranscribed regions, will mean that only 1, in some cases 2 or 3, administrations will be all that is needed to cure, prevent (prevent as used herein can include prophylactic treatment), alleviate (alleviate as used herein can include a complete or partial reduction or relief in a symptom or element of a disorder), or permanently treat the disorder.

In another aspect, the invention features, the pre-natal provision of an RNA described herein, *e.g.*, multiple RNAs described herein.

In another aspect , the invention features, a nucleic acid, *e.g.*, an RNA, or a DNA encoding the RNA, *e.g.*, an RNA described herein, which RNA has been modified to alter its distribution or lifetime, *e.g.*, the RNA has been modified to enhance nuclear localization. In a preferred embodiment the RNA includes one or more phosphorothioate groups. In another preferred embodiment, the RNA includes an RNA nuclear retention signal, *e.g.*, all or an active part of a U6 snRNA retention signal. Such nucleic acids can be used in any of the methods described herein. They are particularly preferred for RNAs that are targeted to non-transcribed regions.

The invention also includes RNAs, and methods of using them, that function as described herein, regardless of whether single or multiple RNAs are administered.

Isolated RNA molecules (double-stranded; single-stranded) can mediate RNAi. The RNA molecules preferably mediate RNAi with respect to an endogenous gene of a subject or to a gene of a pathogen. Such molecules include dsRNAs and siRNAs.

In a preferred embodiment the RNAs are chosen from dsRNAs, including, *e.g.*, a dsRNA described herein, a sense strand, or an antisense strand. Hairpin RNAs can be used. "dsRNA" refers to a double stranded RNA molecule that includes a sufficient number of ribonucleotides such that the dsRNA, or a fragment thereof, can mediate RNAi. The dsRNA can include a strand of any length greater than 17, 18, or 19 nucleotides, or at least 30, 40, or 60 nucleotides. In many embodiments, the dsRNA is sufficiently large that it can be cleaved by an endogenous molecule, *e.g.*, by a Dicer/Argonaut complex, to produce smaller dsRNAs, *e.g.*, siRNAs.

"siRNA or short inhibitory RNA" refers to a dsRNA that is sufficiently short that it does not induce a deleterious interferon response in a human cell, *e.g.*, it has a double-stranded region of less than 60 but preferably less than 50, 40, or 30 nucleotide pairs but can induce RNAi with respect to a target RNA, generally an endogenous or pathogen target RNA. The siRNA is at least partially, and in some embodiments fully, complementary to one strand of the target RNA. It is not necessary that there be perfect complementarity between the siRNA and the target, but the correspondence must be sufficient to enable the RNA to direct sequence specific silencing, *e.g.*, by decreasing transcription of the target gene. While perfect complementarity is often desired other embodiments can include one or more but preferably 6, 5, 4, 3, 2, or fewer mismatches (with respect to the target RNA). The mismatches are most tolerated in the terminal regions and if present are preferably in a terminal region or regions, *e.g.*, within 6, 5, 4, or 3 nucleotides of the 5' and/or 3' terminus of one of the strands. Each siRNA strand of an siRNA duplex can be equal to or less than 30, 25, 24, 23, 22, 21, or 20 nucleotides in length. The strand is preferably at least 19 nucleotides in length. For example, each strand can be between 21 and 25 nt in length. A siRNA duplex contains sense and antisense siRNA, preferable paired to contain an overhang, *e.g.*, one or two 5' or 3' overhangs but preferably a 3' overhang of 2-3 nt. Most embodiments will have a 3' overhang. Preferred siRNAs will have single-stranded overhangs, preferably 3' overhangs, of 1 or preferably 2 or 3 nucleotides in length at each end. The overhangs can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered. 5' ends are preferably phoshorylated. Preferred lengths for the double stranded region is between 15 and 30, most preferably 18, 19, 20, 21, 22,

and 23 nucleotides in length. siRNAs can resemble in length and structure the natural Dicer processed products from long dsRNAs. Embodiments in which the two strands of the siRNA are linked, *e.g.*, covalently linked are also included. Hairpin, or other single strand structures that provide the required double stranded region, and preferably a 3' overhang are also within the invention.

The isolated RNAs described herein, including dsRNAs and siRNAs, mediate silencing of a target gene, *e.g.*, a gene that encodes a protein. For convenience, such a gene is also referred to herein as the gene to be silenced. Such a gene is also referred to as a target gene. In general, the gene to be silenced is an endogenous gene or a pathogenic gene. As used herein, the terms RNA, RNA molecule(s), RNA segment(s) and RNA fragment(s) are used interchangeably to refer to RNA that mediates RNA interference. These terms include double-stranded RNA, single-stranded RNA, isolated RNA (partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA), as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siRNA or internally (at one or more nucleotides of the RNA). Nucleotides in the RNA molecules of the present invention can also comprise non-standard nucleotides, including non-naturally occurring nucleotides or deoxyribonucleotides. Collectively, all such altered RNAs are referred to as analogs or analogs of naturally-occurring RNA. A strand of an siRNA needs only to be sufficiently similar to natural RNA that it has the ability to mediate (mediates) RNAi.

As used herein, the phrase "mediates RNAi" refers to the ability to silence, in a sequence specific manner, a target gene.

The present invention also relates to methods of producing siRNA molecules, *e.g.* siRNA molecules described herein, having the ability to mediate RNAi. These RNA molecules can be formulated for administration to a subject. In other embodiments, the invention features a method of administering a RNA or DNA encoding the RNA *in vitro* (*e.g.*, to a cell, cell line, organ, or tissue). In a preferred embodiment, the cell or tissue can be administered to a subject.

In one aspect, the invention features a method of administering a double-stranded RNA (dsRNA) or DNA encoding the RNA to a subject (*e.g.*, a human

subject or animal subject, *e.g.*, a mouse). The method includes administering a unit dose of the dsRNA, *e.g.*, an siRNA, *e.g.*, an siRNA that (a) is 19-25 nucleotides (nt) long, preferably 21-23 nt, (b) is complementary to a target RNA (*e.g.*, an endogenous or pathogen target RNA), and, optionally, (c) includes at least one 3' overhang 1-5 nt long. In one embodiment, the unit dose is less than 1.4 g per kg of bodyweight, or less than 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.00005 or 0.00001 mg per kg of bodyweight , and less than 100 μmol of RNA (*e.g.*, about $4.4x10^{19}$ copies) per kg of bodyweight or less than 50, 5, 0.15, 0.10, 0.5, 0.05, 0.005, 0.00005 μmol of RNA per kg of bodyweight. The defined amount can be an amount effective to treat or prevent a disease or disorder, *e.g.*, a disease or disorder associated with the target RNA. The unit dose, for example, can be administered by injection (*e.g.*, intravenous or intramuscular), an inhaled dose, or a topical application. Particularly preferred dosages are less than 2, 1, or 0.1 mg/kg of body weight.

In a preferred embodiment, the unit dose is administered less frequently than once a day, *e.g.*, less than every 2, 4, 8 or 30 days. In another embodiment, the unit dose is not administered with a frequency (*e.g.*, not a regular frequency). For example, the unit dose may be administered a single time.

In one embodiment, the effective dose is administered with other traditional therapeutic modalities. In one embodiment, the subject has a viral infection and the modality is an antiviral agent other than an siRNA. In another embodiment, the subject has atherosclerosis and the effective dose of siRNA (or other dsRNA) is administered in combination with, *e.g.*, after surgical intervention, *e.g.*, angioplasty.

In one embodiment, a subject is administered an initial dose and one or more maintenance doses of siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor). The maintenance dose or doses are generally lower than the initial dose, *e.g.*, one-half less of the initial dose. A maintenance regimen can include treating the subject with a dose or doses ranging from 0.01 μg to 1.4 mg/kg of body weight per day, *e.g.*, 10, 1, 0.1, 0.01, 0.001, or 0.00001 mg per kg of bodyweight per day. The maintenance doses are preferably administered no more than once every 5, 10, or 30 days.

In a preferred embodiment, the dsRNA pharmaceutical composition includes a plurality of siRNA species. In another embodiment, each siRNA species has

sequences that are non-overlapping and non-adjacent to another species with respect to a naturally occurring target sequence. In another embodiment, the plurality of siRNA species is specific for different naturally occurring target genes. In another embodiment, the siRNA is allele specific.

The inventors have discovered that RNAs described herein can be administered to mammals, particularly large mammals such as nonhuman primates or humans in a number of ways.

In one embodiment, the administration of the dsRNA composition is parenteral, *e.g.* intravenous (*e.g.*, as a bolus or as a diffusible infusion), intradermal, intraperitoneal, intramuscular, intrathecal, intraventricular, subcutaneous, transmucosal, buccal, sublingual, endoscopic, rectal, oral, vaginal, topical, pulmonary, intranasal, urethral or ocular. Administration can be provided by the subject or by another person, *e.g.*, a health care provider. The medication can be provided in measured doses or in a dispenser that delivers a metered dose. Selected modes of delivery are discussed in more detail below.

The invention provides methods, compositions, and kits, for rectal administration or delivery of RNAs described herein.

Accordingly, an siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) described herein, *e.g.*, a therapeutically effective amount of an siRNA described herein, *e.g.*, an siRNA having a double stranded region of less than 40, and preferably less than 30 nucleotides and having one or two 1-3 nucleotide single strand 3' overhangs can be administered rectally, *e.g.*, introduced through the rectum into the lower or upper colon. This approach is particularly useful in the treatment of, inflammatory disorders, disorders characterized by unwanted cell proliferation, *e.g.*, polyps, or colon cancer.

In some embodiments the medication is delivered to a site in the colon by introducing a dispensing device, *e.g.*, a flexible, camera-guided device similar to that used for inspection of the colon or removal of polyps, which includes means for delivery of the medication.

In one embodiment, the rectal administration of the dsRNA is by means of an enema. The dsRNA of the enema can be dissolved in a saline or buffered solution.

In another embodiment, the rectal administration is by means of a suppository. The suppository can include other ingredients, *e.g.*, an excipient, *e.g.*, cocoa butter or hydropropylmethylcellulose.

The invention also provides methods, compositions, and kits for oral delivery of RNAs described herein.

Accordingly, an siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) described herein, *e.g.*, a therapeutically effective amount of an siRNA described herein, *e.g.*, an siRNA having a double stranded region of less than 40 and preferably less than 30 nucleotides and having one or two 1-3 nucleotide single strand 3' overhangs can be administered orally.

Oral administration can be in the form of tablets, capsules, gel capsules, lozenges, troches or liquid syrups. In a preferred embodiment the composition is applied topically to a surface of the oral cavity.

The invention also provides methods, compositions, and kits for buccal delivery of RNAs described herein.

Accordingly, an siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) described herein, *e.g.*, a therapeutically effective amount of siRNA having a double stranded region of less than 40 and preferably less than 30 nucleotides and having one or two 1-3 nucleotide single strand 3' overhangs can be administered to the buccal cavity. The medication can be sprayed into the buccal cavity or applied directly, *e.g.*, in a liquid, solid, or gel form to a surface in the buccal cavity. This administration is particularly desirable for the treatment of inflammations of the buccal cavity, *e.g.*, the gums or tongue. *E.g.*, in one embodiment, the buccal administration is by spraying into the cavity, *e.g.*, without inhalation, from a dispenser, *e.g.*, a metered dose spray dispenser that dispenses the pharmaceutical composition and a propellant.

The invention also provides methods, compositions, and kits for ocular delivery of RNAs described herein.

Accordingly, one or more siRNAs (or precursors, including, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) described herein, including, *e.g.*, a therapeutically effective amount of

an siRNA described herein, *e.g.*, an siRNA having a double stranded region of less than 40 and preferably less than 30 nucleotides and having one or two 1-3 nucleotide single strand 3' overhangs can be administered to ocular tissue.

The medications can be applied to the surface of the eye or nearby tissue, *e.g.*, the inside of the eyelid. It can be applied topically, *e.g.*, by spraying, in drops, as an eyewash, or an ointment. Administration can be provided by the subject or by another person, *e.g.*, a health care provider. The medication can be provided in measured doses or in a dispenser that delivers a metered dose.

The medication can also be administered to the interior of the eye, and can be introduced by a needle or other delivery device that can introduce it to a selected area or structure.

Ocular treatment is particularly desirable for treating inflammation of the eye or nearby tissue.

The invention also provides methods, compositions, and kits for delivery of RNAs described herein to or through the skin.

Accordingly, an siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) described herein, *e.g.*, a therapeutically effective amount of an siRNA described herein, *e.g.*, an siRNA having a double stranded region of less than 40 and preferably less than 30 nucleotides and one or two 1-3 nucleotide single strand 3' overhangs can be administered directly to the skin.

The medication can be applied topically or delivered in a layer of the skin, *e.g.*, by the use of a microneedle or a battery of microneedles that penetrate into the skin, but preferably not into the underlying muscle tissue.

In one embodiment, the administration of the dsRNA composition is topical. In another embodiment, topical administration delivers the composition to the dermis or epidermis of a subject. In other embodiments the topical administration is in the form of transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids or powders. A composition for topical administration can be formulated as a liposome, micelle, emulsion, or other lipophilic molecular assembly.

In another embodiment, the transdermal administration is applied with at least one penetration enhancer. In other embodiments, the penetration can be enhanced

with iontophoresis, phonophoresis, and sonophoresis. In another aspect, the invention provides methods, compositions, devices, and kits for pulmonary delivery of RNAs described herein.

Accordingly, an siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) described herein, *e.g.*, a therapeutically effective amount of an siRNA described herein, *e.g.*, having a double stranded region of less than 40, preferably less than 30 nucleotides and having one or two 1-3 nucleotide single strand 3' overhangs can be administered to the pulmonary system. Pulmonary administration can be achieved by inhalation or by the introduction of a delivery device into the pulmonary system, *e.g.*, by introducing a delivery device that can dispense the medication.

The preferred method of pulmonary delivery is by inhalation. The medication can be provided in a dispenser that delivers the medication, *e.g.*, wet or dry, in a form sufficiently small such that it can be inhaled. The device can deliver a metered dose of medication. The subject, or another person, can administer the medication.

Pulmonary delivery is effective not only for disorders that directly affect pulmonary tissue, but also for disorders that affect other tissue.

RNA can be formulated as a liquid or nonliquid, *e.g.*, a powder, crystal, or aerosol for pulmonary delivery.

In another aspect, the invention provides methods, compositions, devices, and kits for nasal delivery of RNAs described herein. Accordingly, one or more siRNAs (or precursors, including, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) described herein, including, *e.g.*, a therapeutically effective amount of siRNA, *e.g.*, an siRNA having a double stranded region of less than 40 and preferably less than 30 nucleotides and having one or two 1-3 nucleotide single strand 3' overhangs can be administered nasally. Nasal administration can be achieved by introduction of a delivery device into the nose, *e.g.*, by introducing a delivery device that can dispense the medication.

The preferred method of nasal delivery is by spray, aerosol, liquid, *e.g.*, by drops, of by topical administration to a surface of the nasal cavity. The medication can be provided in a dispenser which delivery of the medication, *e.g.*, wet or dry, in a

form sufficiently small such that it can be inhaled. The device can deliver a metered dose of medication. The subject, or another person, can administer the medication.

Nasal delivery is effective not only for disorders that directly affect nasal tissue, but also for disorders that affect other tissue

RNA can be formulated as a liquid or nonliquid, *e.g.*, a powder, crystal, or for nasal delivery.

In another embodiment, the dsRNA is packaged in a viral natural capsid or in a chemically or enzymatically produced artificial capsid or structure derived therefrom.

In one aspect, of the invention, the dosage of a pharmaceutical composition including dsRNA is administered in order to alleviate the symptoms of a disease state, *e.g.*, cancer or a cardiovascular disease.

In one aspect, gene expression in a subject is modulated by administering a pharmaceutical composition including a dsRNA. In other embodiments, a subject is treated with the pharmaceutical composition by any of the methods mentioned above. In another embodiment, the subject has cancer or is diagnosed with a cancer.

A dsRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) composition can be administered as a liposome. For example, the composition can be prepared by a method that includes: (1) contacting a dsRNA with an amphipathic cationic lipid conjugate in the presence of a detergent; and (2) removing the detergent to form a dsRNA and cationic lipid complex. In one embodiment, the detergent is cholate, deoxycholate, lauryl sarcosine, octanoyl sucrose, CHAPS (3-[(3-cholamidopropyl)-dimethylamine]-2-hydroxyl-1-propane), novel-β-D-glucopyranoside, lauryl dimethylamine oxide, or octylglucoside. The dsRNA can be an siRNA. The method can include preparing a composition that includes a plurality of siRNA, *e.g.*, specific for one or more different endogenous target RNAs. The method can include other features described herein.

In another aspect, a subject is treated by administering a defined amount of a dsRNA composition that is in a powdered form. In one embodiment, the powder is a collection of microparticles. In one embodiment, the powder is a collection of crystalline particles. The composition can include a plurality of siRNA, *e.g.*, specific

for one or more different endogenous target RNAs. The method can include other features described herein.

In one aspect, a subject is treated by administering a defined amount of a dsRNA composition that is prepared by a method that includes spray-drying, i.e. atomizing a liquid solution, emulsion, or suspension, immediately exposing the droplets to a drying gas, and collecting the resulting porous powder particles. The composition can include a plurality of siRNA, *e.g.*, specific for one or more different endogenous target RNAs. The method can include other features described herein.

In one aspect, the dsRNA, *e.g.*, the siRNA, is provided in a powdered, crystallized or other finely divided form, with or without a carrier, *e.g.*, a micro- or nano-particle suitable for inhalation or other pulmonary delivery. In one embodiment, this includes providing an aerosol preparation, *e.g.*, an aerosolized spray-dried composition. The aerosol composition can be provided in and/or dispensed by a metered dose delivery device.

In another aspect, a subject is treated for a condition treatable by inhalation. In one embodiment, this method includes aerosolizing a spray-dried dsRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) composition and inhaling the aerosolized composition. The dsRNA can be an siRNA. The composition can include a plurality of siRNA, *e.g.*, specific for one or more different endogenous target RNAs. The method can include other features described herein.

In another aspect, the invention features a method of treating a subject that includes: administering a composition including an effective/defined amount of siRNA, wherein the composition is prepared by a method that includes spray-drying, lyophilization, vacuum drying, evaporation, fluid bed drying, or a combination of these techniques

In another aspect, the invention features a method that includes: evaluating a parameter related to the abundance of a transcript in a cell of a subject; comparing the evaluated parameter to a reference value; and if the evaluated parameter has a preselected relationship to the reference value (*e.g.*, it is greater), administering one or more siRNAs (or precursors, *e.g.*, larger dsRNAs which can be processed into siRNAs, or DNAs which encode siRNAs or precursors) to the subject. For example,

the parameter can be a direct measure of transcript levels, a measure of a protein level, a disease or disorder symptom or characterization (*e.g.*, rate of cell proliferation and/or tumor mass, viral load).

In another aspect, the invention features a method that includes: evaluating a parameter related to the modification of a target gene or proteins associated with a target gene (*e.g.*, histone proteins), comparing the evaluated parameter relevance to a reference value; and if the evaluated parameter has a preselected relationship to the reference value (*e.g.*, it is greater), administering one or more siRNAs (or precursors, *e.g.*, larger dsRNAs which can be processed into siRNAs, or DNAs which encode siRNAs or precursors) to the subject. For example, the parameter can be a direct measure of modification of a target gene or protein associated with a target gene (*e.g.*, methylation), a measure of a accessibility of a target gene (*e.g.*, by measuring sensitivity of the genomic locus containing the gene to nuclease digestion), or a disease or disorder symptom or characterization (*e.g.*, rate of cell proliferation and/or tumor mass, or viral load).

In another aspect, the invention features a method that includes: administering a first amount of a composition that comprises the siRNAs (or precursors, *e.g.*, larger dsRNAs which can be processed into siRNAs, or DNAs which encode siRNAs or precursors) to a subject, wherein the siRNAs include a strand substantially complementary to a target nucleic acid; evaluating an activity associated with a protein encoded by the target nucleic acid; wherein the evaluation is used to determine if a second amount should be administered. In a preferred embodiment the method includes administering a second amount of the composition, wherein the timing of administration or dosage of the second amount is a function of the evaluating. The method can include other features described herein.

In another aspect, the invention features a method of administering a source of a double-stranded RNA (dsRNA) to a subject. The method includes administering or implanting a source of a dsRNA, *e.g.*, an siRNA, that (a) includes a strand that is 19-25 nucleotides (nt) long, preferably 21-23 nt, (b) is complementary to a target RNA (*e.g.*, an endogenous RNA or a pathogen RNA), and, optionally, (c) includes at least one 3' overhang 1-5 nt long. In one embodiment, the source releases dsRNA over time, *e.g.* the source is a controlled or a slow release source, *e.g.*, a microparticle that

gradually releases the dsRNA. In another embodiment, the source is a pump, *e.g.*, a pump that includes a sensor or a pump that can release one or more unit doses.

In one aspect, the invention features a pharmaceutical composition that includes one or more siRNA molecules including a nucleotide sequence complementary to a gene that can be an endogenous human gene. In one embodiment, the siRNA (a) is 19-25 nucleotides (nt) long, preferably 21-23 nt, (b) is complementary to an endogenous target gene, and, optionally, (c) includes at least one 3' overhang 1-5 nt long. In one embodiment, the pharmaceutical composition can be an emulsion, microemulsion, cream, jelly, or liposome.

In one example the pharmaceutical composition includes siRNAs mixed with a topical delivery agent. The topical delivery agent can be a plurality of microscopic vesicles. The microscopic vesicles can be liposomes. In a preferred embodiment the liposomes are cationic liposomes.

In another aspect, the pharmaceutical composition includes siRNAs admixed with a topical penetration enhancer. In one embodiment, the topical penetration enhancer is a fatty acid. The fatty acid can be arachidonic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-10} alkyl ester, monoglyceride, diglyceride or pharmaceutically acceptable salt thereof.

In another embodiment, the topical penetration enhancer is a bile salt. The bile salt can be cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate, polyoxyethylene-9-lauryl ether or a pharmaceutically acceptable salt thereof.

In another embodiment, the penetration enhancer is a chelating agent. The chelating agent can be EDTA, citric acid, a salicyclate, a N-acyl derivative of collagen, laureth-9, an N-amino acyl derivative of a beta-diketone or a mixture thereof.

In another embodiment, the penetration enhancer is a surfactant, *e.g.*, an ionic or nonionic surfactant. The surfactant can be sodium lauryl sulfate, polyoxyethylene-

9-lauryl ether, polyoxyethylene-20-cetyl ether, a perfluorchemical emulsion or mixture thereof.

In another embodiment, the penetration enhancer can be selected from a group consisting of unsaturated cyclic ureas, 1-alkyl-alkones, 1-alkenylazacyclo-alakanones, steroidal anti-inflammatory agents and mixtures thereof. In yet another embodiment the penetration enhancer can be a glycol, a pyrrol, an azone, or a terpenes.

In one aspect, the invention features a pharmaceutical composition including one or more siRNAs in a form suitable for oral delivery. In one embodiment, oral delivery can be used to deliver a siRNA composition to a cell or a region of the gastro-intestinal tract, *e.g.*, small intestine, colon (*e.g.*, to treat a colon cancer), and so forth. The oral delivery form can be tablets, capsules or gel capsules. In one embodiment, the siRNA of the pharmaceutical composition modulates expression of a cellular adhesion protein, modulates a rate of cellular proliferation, or has biological activity against eukaryotic pathogens or retroviruses. In another embodiment, the pharmaceutical composition includes an enteric material that substantially prevents dissolution of the tablets, capsules or gel capsules in a mammalian stomach. In a preferred embodiment the enteric material is a coating. The coating can be acetate phthalate, propylene glycol, sorbitan monoleate, cellulose acetate trimellitate, hydroxy propyl methyl cellulose phthalate or cellulose acetate phthalate.

In another embodiment, the oral dosage form of the pharmaceutical composition includes a penetration enhancer. The penetration enhancer can be a bile salt or a fatty acid. The bile salt can be ursodeoxycholic acid, chenodeoxycholic acid, and salts thereof. The fatty acid can be capric acid, lauric acid, and salts thereof.

In another embodiment, the oral dosage form of the pharmaceutical composition includes an excipient. In one example the excipient is polyethyleneglycol. In another example the excipient is precirol.

In another embodiment, the oral dosage form of the pharmaceutical composition includes a plasticizer. The plasticizer can be diethyl phthalate, triacetin dibutyl sebacate, dibutyl phthalate or triethyl citrate.

In one aspect, the invention features a pharmaceutical composition including siRNAs and a delivery vehicle. In one embodiment, the siRNAs are (a) is 19-25

nucleotides (nt) long, preferably 21-23 nt, (b) is complementary to an endogenous target gene, and, optionally, (c) includes at least one 3' overhang 1-5 nt long.

In one embodiment, the delivery vehicle can deliver the siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) to a cell by a topical route of administration. The delivery vehicle can be microscopic vesicles. In one example the microscopic vesicles are liposomes. In a preferred embodiment the liposomes are cationic liposomes. In another example the microscopic vesicles are micelles.

In one aspect, the invention features a method for making a pharmaceutical composition, the method including: (1) contacting a siRNA with a amphipathic cationic lipid conjugate in the presence of a detergent; and (2) removing the detergent to form a siRNA and cationic lipid complex.

In another aspect, the invention features a pharmaceutical composition produced by a method including: (1) contacting a siRNA with a amphipathic cationic lipid conjugate in the presence of a detergent; and (2) removing the detergent to form a siRNA and cationic lipid complex. In one embodiment, the detergent is cholate, deoxycholate, lauryl sarcosine, octanoyl sucrose, CHAPS (3-[(3-cholamidopropyl)-dimethylamine]-2-hydroxyl-1-propane), novel-β-D-glucopyranoside, lauryl dimethylamine oxide, or octylglucoside. In another embodiment, the amphipathic cationic lipid conjugate is biodegradable. In yet another embodiment the pharmaceutical composition includes a targeting ligand.

In one aspect, the invention features a pharmaceutical composition including a siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) in an injectable dosage form. In one embodiment, the injectable dosage form of the pharmaceutical composition includes sterile aqueous solutions or dispersions and sterile powders. In a preferred embodiment the sterile solution can include a diluent such as water; saline solution; fixed oils, polyethylene glycols, glycerine, or propylene glycol.

In one aspect, the invention features a pharmaceutical composition including a siRNA in oral dosage form. In one embodiment, the oral dosage form is selected from the group consisting of tablets, capsules and gel capsules. In another embodiment, the pharmaceutical composition includes an enteric material that

substantially prevents dissolution of the tablets, capsules or gel capsules in a mammalian stomach. In a preferred embodiment the enteric material is a coating. The coating can be acetate phthalate, propylene glycol, sorbitan monoleate, cellulose acetate trimellitate, hydroxy propyl methyl cellulose phthalate or cellulose acetate phthalate. In one embodiment, the oral dosage form of the pharmaceutical composition includes a penetration enhancer, *e.g.*, a penetration enhancer described herein.

In another embodiment, the oral dosage form of the pharmaceutical composition includes an excipient. In one example the excipient is polyethyleneglycol. In another example the excipient is precirol.

In another embodiment, the oral dosage form of the pharmaceutical composition includes a plasticizer. The plasticizer can be diethyl phthalate, triacetin dibutyl sebacate, dibutyl phthalate or triethyl citrate.

In one aspect, the invention features a pharmaceutical composition including a siRNA in a rectal dosage form. In one embodiment, the rectal dosage form is an enema. In another embodiment, the rectal dosage form is a suppository.

In one aspect, the invention features a pharmaceutical composition including a siRNA in a vaginal dosage form. In one embodiment, the vaginal dosage form is a suppository. In another embodiment, the vaginal dosage form is a foam, cream, or gel.

In one aspect, the invention features a pharmaceutical composition including a siRNA in a pulmonary or nasal dosage form. In one embodiment, the siRNA is incorporated into a particle, *e.g.*, a macroparticle, *e.g.*, a microsphere. The particle can be produced by spray drying, lyophilization, evaporation, fluid bed drying, vacuum drying, or a combination thereof. The microsphere can be formulated as a suspension, a powder, or an implantable solid.

In one aspect, the invention features a spray-dried siRNA composition suitable for inhalation by a subject, including: (a) a therapeutically effective amount of a siRNA suitable for treating a condition in the subject by inhalation; (b) a pharmaceutically acceptable excipient selected from the group consisting of carbohydrates and amino acids; and (c) optionally, a dispersibility-enhancing amount of a physiologically-acceptable, water-soluble polypeptide.

In one embodiment, the excipient is a carbohydrate. The carbohydrate can be selected from the group consisting of monosaccharides, disaccharides, trisaccharides, and polysaccharides. In a preferred embodiment the carbohydrate is a monosaccharide selected from the group consisting of dextrose, galactose, mannitol, D-mannose, sorbitol, and sorbose. In another preferred embodiment the carbohydrate is a disaccharide selected from the group consisting of lactose, maltose, sucrose, and trehalose.

In another embodiment, the excipient is an amino acid. In one embodiment, the amino acid is a hydrophobic amino acid. In a preferred embodiment the hydrophobic amino acid is selected from the group consisting of alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, and valine. In yet another embodiment the amino acid is a polar amino acid. In a preferred embodiment the amino acid is selected from the group consisting of arginine, histidine, lysine, cysteine, glycine, glutamine, serine, threonine, tyrosine, aspartic acid and glutamic acid.

In one embodiment, the dispersibility-enhancing polypeptide is selected from the group consisting of human serum albumin, α-lactalbumin, trypsinogen, and polyalanine.

In one embodiment, the spray-dried siRNA composition includes particles having a mass median diameter (MMD) of less than 10 microns. In another embodiment, the spray-dried siRNA composition includes particles having a mass median diameter of less than 5 microns. In yet another embodiment the spray-dried siRNA composition includes particles having a mass median aerodynamic diameter (MMAD) of less than 5 microns.

In certain other aspects, the invention provides kits that include a suitable container containing a pharmaceutical formulation of a siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor). In certain embodiments the individual components of the pharmaceutical formulation may be provided in one container. Alternatively, it may be desirable to provide the components of the pharmaceutical formulation separately in two or more containers, *e.g.*, one container for an siRNA preparation, and at least another for a carrier compound. The kit may be packaged in a number of different

configurations such as one or more containers in a single box. The different components can be combined, *e.g.*, according to instructions provided with the kit. The components can be combined according to a method described herein, *e.g.*, to prepare and administer a pharmaceutical composition. The kit can also include a delivery device.

In another aspect, the invention features a device, *e.g.*, an implantable device, wherein the device can dispense or administer a composition that includes an siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor), *e.g.*, an siRNA that silences an endogenous transcript. In one embodiment, the device is coated with the composition. In another embodiment the siRNA is disposed within the device. In another embodiment, the device includes a mechanism to dispense a unit dose of the composition. In other embodiments the device releases the composition continuously, *e.g.*, by diffusion. Exemplary devices include stents, catheters, pumps, artificial organs or organ components (*e.g.*, artificial heart, a heart valve, etc.), and sutures.

As used herein, the term "crystalline" describes a solid having the structure or characteristics of a crystal, i.e., particles of three dimensional structure in which the plane faces intersect at definite angles and in which there is a regular internal structure. The compositions of the invention may have different crystalline forms. Crystalline forms can be prepared by a variety of methods, including, for example, spray drying.

As used herein, "specifically hybridizable" and "complementary" are terms that are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between a compound of the invention and a target gene. Specific binding requires a sufficient degree of complementarity to avoid non-specific binding of the oligomeric compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed. The non-target sequences typically differ by at least 5 nucleotides.

In one embodiment, an siRNA is "sufficiently complementary" or "substantially identical" to a target nucleic acid, *e.g.*, target RNA or DNA, *e.g.*, a

target nucleic acid such that the siRNA silences production of protein encoded by the target gene. In another embodiment, the siRNA is "exactly complementary" or "identical" to a target nucleic acid, *e.g.*, the target nucleic acid and the siRNA can anneal to form a hybrid made exclusively of Watson-Crick basepairs in the region of exact complementarity. A "sufficiently complementary" or "substantially identical" target RNA can include an internal region (*e.g.*, of at least 10 nucleotides) that is exactly complementary to a target RNA. Moreover, in some embodiments, the siRNA specifically discriminates a single-nucleotide difference. In this case, the siRNA only mediates RNAi if exact complementary is found in the region (*e.g.*, within 7 nucleotides of) the single-nucleotide difference. In preferred embodiments two nucleic acids are substantially identical if they share at least 70, 75, 80, 85, more preferably at least 90, 95, or 99% identity.

As used herein, the term "oligonucleotide" refers to a nucleic acid molecule (RNA or DNA) of length less than 300 nucleotides.

In one aspect, the invention features, a method of treating a subject at risk for or afflicted with unwanted cell proliferation, *e.g.*, malignant or nonmalignant cell proliferation. The method includes:

providing one or more siRNAs, including, *e.g.*, a siRNA having a structure described herein, where siRNA is homologous to and can silence, *e.g.*, by decreasing transcription of, a gene that promotes unwanted cell proliferation;

administering the siRNAs to a subject, preferably a human subject,

thereby treating the subject. Preferably a plurality of siRNAs that are substantially identical to nontranscribed regions of genes, including, *e.g.*, a promoter region or other control region, are administered.

In a preferred embodiment the gene is a growth factor or growth factor receptor gene, a kinase, *e.g.*, a protein tyrosine, serine or threonine kinase gene, an adaptor protein gene, a gene encoding a G protein superfamily molecule, or a gene encoding a transcription factor.

In a preferred embodiment the siRNAs silence the PDGF beta gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted PDGF beta expression, *e.g.*, testicular and lung cancers.

In another preferred embodiment the siRNAs silence the Erb-B gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Erb-B expression, *e.g.*, breast cancer.

In a preferred embodiment the siRNAs silence the Src gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Src expression, *e.g.*, colon cancers.

In a preferred embodiment the siRNAs silence the CRK gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted CRK expression, *e.g.*, colon and lung cancers.

In a preferred embodiment the siRNAs silence the GRB2 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted GRB2 expression, *e.g.*, squamous cell carcinoma.

In another preferred embodiment the siRNAs silence the RAS gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted RAS expression, *e.g.*, pancreatic, colon and lung cancers, and chronic leukemia.

In another preferred embodiment the siRNAs silence the MEKK gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted MEKK expression, *e.g.*, squamous cell carcinoma, melanoma or leukemia.

In another preferred embodiment the siRNAs silence the JNK gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted JNK expression, *e.g.*, pancreatic or breast cancers.

In a preferred embodiment the siRNAs silence the RAF gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted RAF expression, *e.g.*, lung cancer or leukemia.

In a preferred embodiment the siRNAs silence the Erk1/2 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Erk1/2 expression, *e.g.*, lung cancer.

In another preferred embodiment the siRNAs silence the PCNA(p21) gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted PCNA expression, *e.g.*, lung cancer.

In a preferred embodiment the siRNAs silence the MYB gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted MYB expression, *e.g.*, colon cancer or chronic myelogenous leukemia.

In a preferred embodiment the siRNAs silence the c-MYC gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted c-MYC expression, *e.g.*, Burkitt's lymphoma or neuroblastoma.

In another preferred embodiment the siRNAs silence the JUN gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted JUN expression, *e.g.*, ovarian, prostate or breast cancers.

In another preferred embodiment the siRNAs silence the FOS gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted FOS expression, *e.g.*, skin or prostate cancers.

In a preferred embodiment the siRNAs silence the BCL-2 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted BCL-2 expression, *e.g.*, lung or prostate cancers or Non-Hodgkin lymphoma.

In a preferred embodiment the siRNAs silence the Cyclin D gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Cyclin D expression, *e.g.*, esophageal and colon cancers.

In a preferred embodiment the siRNAs silence the VEGF gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted VEGF expression, *e.g.*, esophageal and colon cancers.

In a preferred embodiment the siRNAs silence the EGFR gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted EGFR expression, *e.g.*, breast cancer.

In another preferred embodiment the siRNAs silence the Cyclin A gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Cyclin A expression, *e.g.*, lung and cervical cancers.

In another preferred embodiment the siRNAs silence the Cyclin E gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Cyclin E expression, *e.g.*, lung and breast cancers.

In another preferred embodiment the siRNAs silence the WNT-1 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted WNT-1 expression, *e.g.*, basal cell carcinoma.

In another preferred embodiment the siRNAs silence the beta-catenin gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted beta-catenin expression, *e.g.*, adenocarcinoma or hepatocellular carcinoma.

In another preferred embodiment the siRNAs silence the c-MET gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted c-MET expression, *e.g.*, hepatocellular carcinoma.

In another preferred embodiment the siRNAs silence the PKC gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted PKC expression, *e.g.*, breast cancer.

In a preferred embodiment the siRNAs silence the NFKB gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted NFKB expression, *e.g.*, breast cancer.

In a preferred embodiment the siRNAs silence the STAT3 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted STAT3 expression, *e.g.*, prostate cancer.

In another preferred embodiment the siRNAs silence the survivin gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted survivin expression, *e.g.*, cervical or pancreatic cancers.

In another preferred embodiment the siRNAs silence the Her2/Neu gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Her2/Neu expression, *e.g.*, breast cancer.

In another preferred embodiment the siRNAs silence the topoisomerase I gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted topoisomerase I expression, *e.g.*, ovarian and colon cancers.

In a preferred embodiment the siRNAs silence the topoisomerase II alpha gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted topoisomerase II expression, *e.g.*, breast and colon cancers.

In a preferred embodiment the siRNAs silence mutations in the p73 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted p73 expression, *e.g.*, colorectal adenocarcinoma.

In a preferred embodiment the siRNAs silence mutations in the p21(WAF1/CIP1) gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted p21(WAF1/CIP1) expression, *e.g.*, liver cancer.

In a preferred embodiment the siRNAs silence mutations in the p27(KIP1) gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted p27(KIP1) expression, *e.g.*, liver cancer.

In a preferred embodiment the siRNAs silence mutations in the PPM1D gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted PPM1D expression, *e.g.*, breast cancer.

In a preferred embodiment the siRNAs silence mutations in the RAS gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted RAS expression, *e.g.*, breast cancer.

In another preferred embodiment the siRNAs silence mutations in the caveolin I gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted caveolin I expression, *e.g.*, esophageal squamous cell carcinoma.

In another preferred embodiment the siRNAs silence mutations in the MIB I gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted MIB I expression, *e.g.*, male breast carcinoma (MBC).

In another preferred embodiment the siRNAs silence mutations in the MTAI gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted MTAI expression, *e.g.*, ovarian carcinoma.

In another preferred embodiment the siRNAs silence mutations in the M68 gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted M68 expression, *e.g.*, human adenocarcinomas of the esophagus, stomach, colon, and rectum.

In preferred embodiments the siRNAs silence mutations in tumor suppressor genes, and thus can be used as a method to promote apoptotic activity in combination with chemotherapeutics.

In a preferred embodiment the siRNAs silence mutations in the p53 tumor suppressor gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted p53 expression, *e.g.*, gall bladder, pancreatic and lung cancers.

In a preferred embodiment the siRNAs silence mutations in the p53 family member DN-p63, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted DN-p63 expression, *e.g.*, squamous cell carcinoma.

In a preferred embodiment the siRNAs silence mutations in the pRb tumor suppressor gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted pRb expression, *e.g.*, oral squamous cell carcinoma.

In a preferred embodiment the siRNAs silence mutations in the APC1 tumor suppressor gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted APC1 expression, *e.g.*, colon cancer.

In a preferred embodiment the siRNAs silence mutations in the BRCA1 tumor suppressor gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted BRCA1 expression, *e.g.*, breast cancer.

In a preferred embodiment the siRNAs silence mutations in the PTEN tumor suppressor gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted PTEN expression, *e.g.*, hamartomas, gliomas, and prostate and endometrial cancers.

In a preferred embodiment the siRNAs silence MLL fusion genes, *e.g.*, MLL-AF9, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted MLL fusion gene expression, *e.g.*, acute leukemias.

In another preferred embodiment the siRNAs silence the BCR/ABL fusion gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted BCR/ABL fusion gene expression, *e.g.*, acute and chronic leukemias.

In another preferred embodiment the siRNAs silence the TEL/AML1 fusion gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted TEL/AML1 fusion gene expression, *e.g.*, childhood acute leukemia.

In another preferred embodiment the siRNAs silence the EWS/FLI1 fusion gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted EWS/FLI1 fusion gene expression, *e.g.*, Ewing Sarcoma.

In another preferred embodiment the siRNAs silence the TLS/FUS1 fusion gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted TLS/FUS1 fusion gene expression, *e.g.*, Myxoid liposarcoma.

In another preferred embodiment the siRNAs silence the PAX3/FKHR fusion gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted PAX3/FKHR fusion gene expression, *e.g.*, Myxoid liposarcoma.

In another preferred embodiment the siRNAs silence the AML1/ETO fusion gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted AML1/ETO fusion gene expression, *e.g.*, acute leukemia.

In another aspect, the invention features, a method of treating a subject, *e.g.*, a human, at risk for or afflicted with a disease or disorder that may benefit by angiogenesis inhibition *e.g.*, cancer. The method includes:

providing one or more siRNAs, including, *e.g.*, an siRNA having a structure described herein, which siRNA is homologous to and can silence, *e.g.*, by inhibiting transcription of a gene that mediates angiogenesis; administering the siRNAs to a subject,

thereby treating the subject.

In a preferred embodiment the siRNAs silence the alpha v-integrin gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted alpha V integrin, *e.g.*, brain tumors or tumors of epithelial origin.

In a preferred embodiment the siRNAs silence the Flt-1 receptor gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted Flt-1 receptors, *e.g.* Cancer and rheumatoid arthritis.

In a preferred embodiment the siRNAs silence the tubulin gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted tubulin, *e.g.* Cancer and retinal neovascularization.

In a preferred embodiment the siRNAs silence the tubulin gene, and thus can be used to treat a subject having or at risk for a disorder characterized by unwanted tubulin, *e.g.* Cancer and retinal neovascularization.

In another aspect, the invention features a method of treating a subject infected with a virus or at risk for or afflicted with a disorder or disease associated with a viral infection. The method includes:

providing one or more siRNA, including, *e.g.*, an siRNA having a structure described herein, which siRNA is homologous to and can silence, *e.g.*, by decreasing transcription, a viral gene or a cellular gene which mediates viral function, *e.g.*, entry or growth; administering the siRNAs to a subject, preferably a human subject, thereby treating the subject. Preferably, at least one of the siRNAs that is administered is substantially identical to a nontranscribed region of the target gene.

Thus, the invention provides for a method of treating patients infected by the Human Papilloma Virus (HPV) or at risk for or afflicted with a disorder mediated by HPV, *e.g*, cervical cancer. HPV is linked to 95% of cervical carcinomas and thus an antiviral therapy is an attractive method to treat these cancers and other symptoms of viral infection.

In a preferred embodiment, the expression of a HPV gene is reduced. In another preferred embodiment, the HPV gene is one of the group of E2, E6, or E7.

In a preferred embodiment the expression of a human gene that is required for HPV replication is reduced.

The invention also includes a method of treating patients infected by the Human Immunodeficiency Virus (HIV) or at risk for or afflicted with a disorder mediated by HIV, *e.g.*, Acquired Immune Deficiency Syndrome (AIDS).

In a preferred embodiment, the expression of a human gene that is required for HIV infection or replication is reduced. In another preferred embodiment, the gene is CCR5, CD4 or Tsg101.

In a preferred embodiment the expression of a human gene that is required for HIV replication is reduced. In another preferred embodiment, the gene is CD4 or Tsg101.

The invention also includes a method for treating patients infected by the Hepatitis B Virus (HBV) or at risk for or afflicted with a disorder mediated by HBV, *e.g.*, cirrhosis and heptocellular carcinoma.

In a preferred embodiment, the expression of a HBV gene is reduced. In another preferred embodiment, the targeted HBV gene encodes one of the group of the tail region of the HBV core protein, the pre-cregious (pre-c) region, or the cregious (c) region. In another preferred embodiment, a targeted HBV-RNA sequence is comprised of the poly(A) tail.

In preferred embodiment the expression of a human gene that is required for HBV replication is reduced.

The invention also provides for a method of treating patients infected by the Hepatitis A Virus (HAV), or at risk for or afflicted with a disorder mediated by HAV.

In a preferred embodiment the expression of a human gene that is required for HAV replication is reduced.

The present invention provides for a method of treating patients infected by the Hepatitis C Virus (HCV), or at risk for or afflicted with a disorder mediated by HCV, *e.g.*, cirrhosis.

In a preferred embodiment, the expression of a HCV gene is reduced.

In another preferred embodiment the expression of a human gene that is required for HCV replication is reduced.

The present invention also provides for a method of treating patients infected by the any of the group of Hepatitis Viral strains comprising hepatitis D, E, F, G, or H, or patients at risk for or afflicted with a disorder mediated by any of these strains of hepatitis.

In a preferred embodiment, the expression of a Hepatitis, D, E, F, G, or H gene is reduced.

In another preferred embodiment the expression of a human gene that is required for hepatitis D, E, F, G or H replication is reduced.

Methods of the invention also provide for treating patients infected by the Respiratory Syncytial Virus (RSV) or at risk for or afflicted with a disorder mediated by RSV, *e.g*, lower respiratory tract infection in infants and childhood asthma, pneumonia and other complications, *e.g.*, in the elderly.

In a preferred embodiment, the expression of a RSV gene is reduced. In another preferred embodiment, the targeted HBV gene encodes one of the group of genes N, L, or P.

In a preferred embodiment the expression of a human gene that is required for RSV replication is reduced.

Methods of the invention provide for treating patients infected by the Herpes Simplex Virus (HSV) or at risk for or afflicted with a disorder mediated by HSV, *e.g*, genital herpes and cold sores as well as life-threatening or sight-impairing disease mainly in immunocompromised patients.

In a preferred embodiment, the expression of a HSV gene is reduced. In another preferred embodiment, the targeted HSV gene encodes DNA polymerase or the helicase-primase.

In a preferred embodiment the expression of a human gene that is required for HSV replication is reduced.

The invention also provides a method for treating patients infected by the herpes Cytomegalovirus (CMV) or at risk for or afflicted with a disorder mediated by CMV, *e.g.*, congenital virus infections and morbidity in immunocompromised patients.

In a preferred embodiment, the expression of a CMV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for CMV replication is reduced.

Methods of the invention also provide for a method of treating patients infected by the herpes Epstein Barr Virus (EBV) or at risk for or afflicted with a disorder mediated by EBV, *e.g.*, NK/T-cell lymphoma, non-Hodgkin lymphoma, and Hodgkin disease.

In a preferred embodiment, the expression of a EBV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for EBV replication is reduced.

Methods of the invention also provide for treating patients infected by Kaposi's Sarcoma-associated Herpes Virus (KSHV), also called human herpesvirus 8, or patients at risk for or afflicted with a disorder mediated by KSHV, *e.g.*, Kaposi's

sarcoma, multicentric Castleman's disease and AIDS-associated primary effusion lymphoma.

In a preferred embodiment, the expression of a KSHV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for KSHV replication is reduced.

The invention also includes a method for treating patients infected by the JC Virus (JCV) or a disease or disorder associated with this virus, *e.g.*, progressive multifocal leukoencephalopathy (PML).

In a preferred embodiment, the expression of a JCV gene is reduced.

In preferred embodiment the expression of a human gene that is required for JCV replication is reduced.

Methods of the invention also provide for treating patients infected by the myxovirus or at risk for or afflicted with a disorder mediated by myxovirus, *e.g.*, influenza.

In a preferred embodiment, the expression of a myxovirus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for myxovirus replication is reduced.

Methods of the invention also provide for treating patients infected by the rhinovirus or at risk for of afflicted with a disorder mediated by rhinovirus, *e.g.*, the common cold.

In a preferred embodiment, the expression of a rhinovirus gene is reduced.

In preferred embodiment the expression of a human gene that is required for rhinovirus replication is reduced.

Methods of the invention also provide for treating patients infected by the coronavirus or at risk for of afflicted with a disorder mediated by coronavirus, *e.g.*, the common cold.

In a preferred embodiment, the expression of a coronavirus gene is reduced.

In preferred embodiment the expression of a human gene that is required for coronavirus replication is reduced.

Methods of the invention also provide for treating patients infected by the flavivirus West Nile or at risk for or afflicted with a disorder mediated by West Nile Virus.

In a preferred embodiment, the expression of a West Nile Virus gene is reduced. In another preferred embodiment, the West Nile Virus gene is one of the group comprising E, NS3, or NS5.

In a preferred embodiment the expression of a human gene that is required for West Nile Virus replication is reduced.

Methods of the invention also provide for treating patients infected by the St. Louis Encephalitis flavivirus, or at risk for or afflicted with a disease or disorder associated with this virus, *e.g.*, viral haemorrhagic fever or neurological disease.

In a preferred embodiment, the expression of a St. Louis Encephalitis gene is reduced.

In a preferred embodiment the expression of a human gene that is required for St. Louis Encephalitis virus replication is reduced.

Methods of the invention also provide for treating patients infected by the Tick-borne encephalitis flavivirus, or at risk for or afflicted with a disorder mediated by Tick-borne encephalitis virus, *e.g.*, viral haemorrhagic fever and neurological disease.

In a preferred embodiment, the expression of a Tick-borne encephalitis virus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Tick-borne encephalitis virus replication is reduced.

Methods of the invention also provide for methods of treating patients infected by the Murray Valley encephalitis flavivirus, which commonly results in viral haemorrhagic fever and neurological disease.

In a preferred embodiment, the expression of a Murray Valley encephalitis virus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Murray Valley encephalitis virus replication is reduced.

The invention also includes methods for treating patients infected by the dengue flavivirus, or a disease or disorder associated with this virus, *e.g.*, dengue haemorrhagic fever.

In a preferred embodiment, the expression of a dengue virus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for dengue virus replication is reduced.

Methods of the invention also provide for treating patients infected by the Simian Virus 40 (SV40) or at risk for or afflicted with a disorder mediated by SV40, *e.g.*, tumorigenesis.

In a preferred embodiment, the expression of a SV40 gene is reduced.

In a preferred embodiment the expression of a human gene that is required for SV40 replication is reduced.

The invention also includes methods for treating patients infected by the Human T Cell Lymphotropic Virus (HTLV), or a disease or disorder associated with this virus, *e.g.*, leukemia and myelopathy.

In a preferred embodiment, the expression of a HTLV gene is reduced. In another preferred embodiment the HTLV1 gene is the Tax transcriptional activator.

In a preferred embodiment the expression of a human gene that is required for HTLV replication is reduced.

Methods of the invention also provide for treating patients infected by the Moloney-Murine Leukemia Virus (Mo-MuLV) or at risk for or afflicted with a disorder mediated by Mo-MuLV, *e.g.*, T-cell leukemia.

In a preferred embodiment, the expression of a Mo-MuLV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Mo-MuLV replication is reduced.

Methods of the invention also provide for treating patients infected by the encephalomyocarditis virus (EMCV) or at risk for or afflicted with a disorder mediated by EMCV, *e.g.* myocarditis. EMCV leads to myocarditis in mice and pigs and is capable of infecting human myocardial cells. This virus is therefore a concern for patients undergoing xenotransplantation.

In a preferred embodiment, the expression of a EMCV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for EMCV replication is reduced.

The invention also includes a method for treating patients infected by the measles virus (MV) or at risk for or afflicted with a disorder mediated by MV, *e.g.* measles.

In a preferred embodiment, the expression of a MV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for MV replication is reduced.

The invention also includes a method for treating patients infected by the Vericella zoster virus (VZV) or at risk for or afflicted with a disorder mediated by VZV, *e.g.* chicken pox or shingles (also called zoster).

In a preferred embodiment, the expression of a VZV gene is reduced.

In a preferred embodiment the expression of a human gene that is required for VZV replication is reduced.

The invention also includes a method for treating patients infected by an adenovirus or at risk for or afflicted with a disorder mediated by an adenovirus, *e.g.* respiratory tract infection.

In a preferred embodiment, the expression of an adenovirus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for adenovirus replication is reduced.

The invention includes a method for treating patients infected by a yellow fever virus (YFV) or at risk for or afflicted with a disorder mediated by a YFV, *e.g.* respiratory tract infection.

In a preferred embodiment, the expression of a YFV gene is reduced. In another preferred embodiment, the preferred gene is one of a group that includes the E, NS2A, or NS3 genes.

In a preferred embodiment the expression of a human gene that is required for YFV replication is reduced.

Methods of the invention also provide for treating patients infected by the poliovirus or at risk for or afflicted with a disorder mediated by poliovirus, *e.g.*, polio.

In a preferred embodiment, the expression of a poliovirus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for poliovirus replication is reduced.

Methods of the invention also provide for treating patients infected by a poxvirus or at risk for or afflicted with a disorder mediated by a poxvirus, *e.g.*, smallpox

In a preferred embodiment, the expression of a poxvirus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for poxvirus replication is reduced.

In another, aspect the invention features methods of treating a subject infected with a pathogen, *e.g.*, a bacterial, amoebic, parasitic, or fungal pathogen. The method includes:

providing one or more siRNAs, including, *e.g.*, a siRNA having a structure described herein, where siRNA is homologous to and can silence, *e.g.*, by decreasing transcription of a pathogen gene;

administering the siRNA to a subject, preferably a human subject, thereby treating the subject.

The target gene can be one involved in growth, cell wall synthesis, protein synthesis, transcription, energy metabolism, *e.g.*, the Krebs cycle, or toxin production.

Thus, the present invention provides for a method of treating patients infected by a plasmodium that causes malaria.

In a preferred embodiment, the expression of a plasmodium gene is reduced. In another preferred embodiment, the gene is apical membrane antigen 1 (AMA1).

In a preferred embodiment the expression of a human gene that is required for plasmodium replication is reduced.

The invention also includes methods for treating patients infected by the Mycobacterium ulcerans, or a disease or disorder associated with this pathogen, *e.g.* Buruli ulcers.

In a preferred embodiment, the expression of a Mycobacterium ulcerans gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Mycobacterium ulcerans replication is reduced.

The invention also includes methods for treating patients infected by the Mycobacterium tuberculosis, or a disease or disorder associated with this pathogen, *e.g.* tuberculosis.

In a preferred embodiment, the expression of a Mycobacterium tuberculosis gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Mycobacterium tuberculosis replication is reduced.

The invention also includes methods for treating patients infected by the Mycobacterium leprae, or a disease or disorder associated with this pathogen, *e.g.* leprosy.

In a preferred embodiment, the expression of a Mycobacterium leprae gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Mycobacterium leprae replication is reduced.

The invention also includes methods for treating patients infected by the bacteria Staphylococcus aureus, or a disease or disorder associated with this pathogen, *e.g.* infections of the skin and mucous membranes.

In a preferred embodiment, the expression of a Staphylococcus aureus gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Staphylococcus aureus replication is reduced.

The invention also includes methods for treating patients infected by the bacteria Streptococcus pneumonia, or a disease or disorder associated with this pathogen, *e.g.* pneumonia or childhood lower respiratory tract infection.

In a preferred embodiment, the expression of a Streptococcus pneumonia gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Streptococcus pneumonia replication is reduced.

The invention also includes methods for treating patients infected by the bacteria Streptococcus pyogenes, or a disease or disorder associated with this pathogen, *e.g.* Strep throat or Scarlet fever.

In a preferred embodiment, the expression of a Streptococcus pyogenes gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Streptococcus pyogenes replication is reduced.

The invention also includes methods for treating patients infected by the bacteria Chlamydia pneumonia, or a disease or disorder associated with this pathogen, *e.g.* pneumonia or childhood lower respiratory tract infection

In a preferred embodiment, the expression of a Chlamydia pneumonia gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Chlamydia pneumonia replication is reduced.

The invention also includes methods for treating patients infected by the bacteria Mycoplasma pneumonia, or a disease or disorder associated with this pathogen, *e.g.* pneumonia or childhood lower respiratory tract infection

In a preferred embodiment, the expression of a Mycoplasma pneumonia gene is reduced.

In a preferred embodiment the expression of a human gene that is required for Mycoplasma pneumonia replication is reduced.

In one aspect, the invention features, a method of treating a subject, *e.g.*, a human, at risk for or afflicted with a disease or disorder characterized by an unwanted immune response, *e.g.*, an inflammatory disease or disorder, or an autoimmune disease or disorder. The method includes:

providing one or more siRNAs, including, *e.g.*, an siRNA having a structure described herein, which siRNA is homologous to and can silence, *e.g.*, by decreasing transcription, a gene which mediates an unwanted immune response;

administering the siRNAs to a subject, thereby treating the subject.

In a preferred embodiment the disease or disorder is an ischemia or reperfusion injury, *e.g.*, ischemia or reperfusion injury associated with acute myocardial infarction, unstable angina, cardiopulmonary bypass, surgical intervention *e.g.*, angioplasty, *e.g.*, percutaneous transluminal coronary angioplasty, the response to a transplanted organ or tissue, *e.g.*, transplanted cardiac or vascular tissue; or thrombolysis.

In a preferred embodiment the disease or disorder is restenosis, *e.g.*, restenosis associated with surgical intervention *e.g.*, angioplasty, *e.g.*, percutaneous transluminal coronary angioplasty.

In a preferred embodiment the disease or disorder is Inflammatory Bowel Disease, *e.g.*, Crohn Disease or Ulcerative Colitis.

In a preferred embodiment the disease or disorder is inflammation associated with an infection or injury.

In a preferred embodiment the disease or disorder is asthma, lupus, multiple sclerosis, diabetes, *e.g.*, type II diabetes, arthritis, *e.g.*, rheumatoid or psoriatic.

In particularly preferred embodiments the siRNAs silence an integrin or co-ligand thereof, *e.g.*, VLA4, VCAM, ICAM.

In particularly preferred embodiments the siRNAs silence a selectin or co-ligand thereof, *e.g.*, P-selectin, E-selectin (ELAM), I-selectin, P-selectin glycoprotein-1 (PSGL-1).

In particularly preferred embodiments the siRNAs silence a component of the complement system, *e.g.*, C3, C5, C3aR, C5aR, C3 convertase, C5 convertase.

In particularly preferred embodiments the siRNAs silence a chemokine or receptor thereof, *e.g.*, TNFI, TNFJ, IL-1I, IL-1J, IL –2, IL-2R, IL-4, IL-4R, IL-5, IL-6, IL-8, TNFRI, TNFRII, IgE, SCYA11, CCR3.

In other embodiments the siRNAs silence GCSF, Gro1, Gro2, Gro3, PF4, MIG, Pro-Platelet Basic Protein (PPBP), MIP-1I, MIP-1J, RANTES, MCP-1, MCP-2, MCP-3, CMBKR1, CMBKR2, CMBKR3, CMBKR5, AIF-1, I-309.

In one aspect, the invention features, a method of treating a subject, *e.g.*, a human, at risk for or afflicted with acute pain or chronic pain. The method includes:

providing one or more siRNAs, including, *e.g.*, an siRNA having a structure described herein, which siRNA is homologous to and can silence, *e.g.*, by decreasing transcription, a gene which mediates the processing of pain;

administering the siRNAs to a subject, thereby treating the subject.

In particularly preferred embodiments the siRNA silences a component of an ion channel.

In particularly preferred embodiments the siRNA silences a neurotransmitter receptor or ligand.

In one aspect, the invention features, a method of treating a subject, *e.g.*, a human, at risk for or afflicted with a neurological disease or disorder. The method includes:

providing one or more siRNAs, including, *e.g.*, an siRNA having a structure described herein, which siRNA is homologous to and can silence, *e.g.*, by decreasing transcription, a gene which mediates a neurological disease or disorder;

administering the siRNAs to a subject, thereby treating the subject.

In a preferred embodiment the disease or disorder is Alzheimer Disease or Parkinson Disease.

In particularly preferred embodiments the siRNA silences an amyloid-family gene, *e.g.*, APP; a presenilin gene, *e.g.*, PSEN1 and PSEN2, or I-synuclein.

In a preferred embodiment the disease or disorder is a neurodegenerative trinucleotide repeat disorder, *e.g.*, Huntington disease, dentatorubral pallidoluysian atrophy or a spinocerebellar ataxia, *e.g.*, SCA1, SCA2, SCA3 (Machado-Joseph disease), SCA7 or SCA8.

In particularly preferred embodiments the siRNA silences HD, DRPLA, SCA1, SCA2, MJD1, CACNL1A4, SCA7, SCA8.

In another aspect, the invention includes vectors, *e.g.*, expression vectors, which encode siRNAs, *e.g.*, siRNAs homologous with a gene disclosed herein. These include vectors which can express one or both strands of one or more siRNAs; a composition which includes a first vector which encodes a first strand of an siRNA and a second vector which encodes a second strand of an siRNA; a vector which encodes a hairpin siRNA, *e.g.*, a hairpin which upon cleavage provides both strands of an siRNA. The vector can also encode, and preferably express, a protein, *e.g.*, a protein active in siRNA metabolism or function, *e.g.*, Dicer or Ago2.

In a preferred embodiment the vector or delivery method, *e.g.*, a virus, is selected such that the siRNA integrates into a preselected site, *e.g.*, a site which will result in modulated, *e.g.*, inducible or controlled expression, *e.g.*, by a polIII promoter or by a polII promoter containing temporal, developmental, disease-state, or tissue specific promoter elements. In other embodiments the expression of an siRNA strand is driven by a temporal, developmental, disease-state, or tissue specific promoter, *e.g.*, a polII promoter containing temporal, developmental, disease-state or tissue specific elements.

The present invention also relates to a method of mediating RNA interference of a gene in a cell or organism (*e.g.*, mammal such as a mouse or a human). In one embodiment, RNA of about 21 to about 23 nt which targets the gene for silencing is introduced into the cell or organism. The cell or organism is maintained under conditions under which modulation of gene expression occurs, thereby mediating RNA interference of the gene in the cell or organism. The cell or organism can be

one in which RNAi occurs as the cell or organism is obtained or a cell or organism can be one that has been modified so that RNAi occurs (*e.g.*, by addition of components obtained from a cell or cell extract that mediate RNAi or activation of endogenous components). As used herein, the term "cell or organism in which RNAi occurs" includes both a cell or organism in which RNAi occurs as the cell or organism is obtained, or a cell or organism that has been modified so that RNAi occurs. In another embodiment, the method of mediating RNA interference of a gene in a cell comprises combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNAs of about 21 to about 23 nucleotides. 21 to 23 nt RNA is then isolated and introduced into the cell or organism. The cell or organism is maintained under conditions in which modulation of gene expression occurs, thereby mediating RNA interference of the gene in the cell or organism. As described for the previous embodiment, the cell or organism is one in which RNAi occurs naturally (in the cell or organism as obtained) or has been modified in such a manner that RNAi occurs. 21 to 23 nt RNAs can also be produced by other methods, such as chemical synthetic methods or recombinant DNA techniques.

The present invention also relates to biochemical components of a cell, such as a Drosophila cell, that process dsRNA to RNA of about 21 to about 23 nucleotides. In addition, biochemical components of a cell that are involved in targeting of a target gene by RNA of about 21 to about 23 nucleotides are the subject of the present invention. In both embodiments, the biochemical components can be obtained from a cell in which they occur or can be produced by other methods, such as chemical synthesis or recombinant DNA methods.

As used herein, the term "isolated" includes materials (*e.g.*, biochemical components, RNA) obtained from a source in which they occur and materials produced by methods such as chemical synthesis or recombinant nucleic acid (DNA, RNA) methods.

The present invention also relates to a method for knocking down (partially or completely) the targeted gene, thus providing an alternative to presently available methods of knocking down (or out) a gene or genes. This method of knocking down

gene expression can be used therapeutically or for research (*e.g.*, to generate models of disease states, to examine the function of a gene, to assess whether an agent acts on a gene, to validate targets for drug discovery). In those instances in which gene function is eliminated, the resulting cell or organism can also be referred to as a knockout. One embodiment of the method of producing knockdown cells and organisms comprises introducing into a cell or organism in which a gene (referred to as a targeted gene) is to be knocked down, RNA of about 21 to about 23 nt that targets the gene and maintaining the resulting cell or organism under conditions under which RNAi occurs, resulting in reduced expression of the targeted gene, thereby producing knockdown cells or organisms. Knockdown cells and organisms produced by the present method are also the subject of this invention.

The present invention also relates to a method of examining or assessing the function of a gene in a cell or organism. In one embodiment, RNA of about 21 to about 23 nt which targets the gene for inhibition of transcription is introduced into a cell or organism in which RNAi occurs. The cell or organism is referred to as a test cell or organism. The test cell or organism is maintained under conditions under which modulation of gene expression occurs. The phenotype of the test cell or organism is then observed and compared to that of an appropriate control cell or organism, such as a corresponding cell or organism that is treated in the same manner except that the target (specific) gene is not targeted. A 21 to 23 nt RNA that does not target the gene can be introduced into the control cell or organism in place of the RNA introduced into the test cell or organism, although it is not necessary to do so. A difference between the phenotypes of the test and control cells or organisms provides information about the function of the targeted gene. In another embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract that mediates RNAi, such as the soluble extract derived from Drosophila embryo described herein, under conditions in which the double-stranded RNA is processed to generate RNA of about 21 to about 23 nucleotides. The RNA of about 21 to about 23 nucleotides is isolated and then introduced into a cell or organism in which RNAi occurs (test cell or test organism). The test cell or test organism is maintained under conditions under which modulation of expression of the target gene occurs. The phenotype of the test cell or organism is then observed and compared to

that of an appropriate control, such as a corresponding cell or organism that is treated in the same manner as the test cell or organism except that the target gene is not targeted. A difference between the phenotypes of the test and control cells or organisms provides information about the function of the targeted gene. The information provided may be sufficient to identify (define) the function of the gene or may be used in conjunction with information obtained from other assays or analyses to do so.

Also the subject of the present invention is a method of validating whether an agent acts on a gene. In this method, RNAs of from about 21 to about 23 nucleotides that target the gene to be silenced are introduced into a cell or organism in which RNAi occurs. The cell or organism (which contains the introduced RNA) is maintained under conditions under which modulation of gene expression occurs, and the agent is introduced into the cell or organism. Whether the agent has an effect on the cell or organism is determined; if the agent has no effect on the ell or organism, then the agent acts on the gene.

The present invention also relates to a method of validating whether a gene product is a target for drug discovery or development. RNAs of from about 21 to about 23 nucleotides that target the gene for modulation of expression are introduced into a cell or organism. The cell or organism is maintained under conditions in which modulation of transcription occurs, resulting in decreased expression of the gene. Whether decreased expression of the gene has an effect on the cell or organism is determined, where in decreased expression of the gene has an effect, then the gene product is a target for drug discovery or development.

The present invention also encompasses a method of treating a disease or condition associated with the presence of a protein in an individual comprising administering to the individual RNAs of from about 21 to about 23 nucleotides which target the gene encoding the protein for modulation. As a result, the protein is not produced or is not produced to the extent it would be in the absence of the treatment.

Also encompassed by the present invention is a gene identified by the sequencing of endogenous 21 to 23 nucleotide RNA molecules that mediate RNA interference.

Also encompassed by the present invention is a method of identifying target sites within a gene that are particularly suitable for RNAi as well as a method of assessing the ability of 21-23 nt RNAs to mediate RNAi.

The details of one or more embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from this description, and from the claims. This application incorporates all cited references, patents, and patent applications by references in their entirety for all purposes.

DETAILED DESCRIPTION

Double-stranded (dsRNA) directs the sequence-specific silencing of genes. The silencing can occur by modulating gene expression at the level of transcription. RNA may stimulate the covalent modification of target DNA sequences or of proteins associated with target sequences.

It has been demonstrated that 21-23 nt fragments of dsRNA are sequence-specific mediators of gene silencing, *e.g.*, by causing inhibition of transcription. A molecular signal, which may be merely the specific length of the fragments, present in these 21-23 nt fragments recruits cellular factors that mediate RNAi. Described herein are methods of modulating, preferably decreasing, gene expression, *e.g.*, in a mammal, *e.g.*, a human by, for administering one or more RNAs, one of which is substantially identical to a nontranscribed region, *e.g.*, a 3' or 5' control region, *e.g.*, promoter sequence of a target gene, and both of which target the same gene. In other aspects, DNAs which encode the RNAs can be administered. Also included are methods for preparing and administering these RNA fragments, and other siRNAs, and their use for specifically inactivating gene function. The use of siRNAs (or recombinantly produced or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific genes for silencing in mammalian cells. In addition, longer dsRNA fragments can also be used.

One or more RNAs which are substantially identical to a coding region or non-coding region of a gene may be administered. As used herein, the term "gene" is defined in its broadest context as a nucleic acid including an open reading frame (including exons and introns), 5' and 3' untranslated regions, and untranscribed

transcription control elements (including 5' and 3' untranscribed sequences (UTS)). Coding sequences specify the RNA-encoding sequences . Noncoding sequences include untranscribed regulatory regions, *e.g.*, promoter, enhancer, repressor, and insulator regions. . A promoter is a DNA sequence which has the ability to induce transcription of a DNA sequence adjacent to it. A promoter can include a core region containing CAAT or TATA nucleotide sequences and surrounding DNA sequences. A promoter is located 5' of the RNA-encoding sequence of the gene and induces transcription of RNA sequences that are located 3' of the promoter sequence. Methods of isolating promoter sequences and other regulatory sequences are known in the art. See, for example, US Pat. No. 6,452,066, US Pat. No. 6,437,221, WO9604390.

Although, in mammalian cells, long dsRNAs can induce the interferon response which is frequently deleterious, siRNAs do not trigger the interferon response, at least not to an extent that is deleterious to the cell and host. In particular, the length of the RNA strands in an siRNA can be less than 31, 30, 28, 25, or 23 nt, *e.g.*, sufficiently short to avoid inducing a deleterious interferon response. Thus, the administration of a composition of siRNA (*e.g.*, formulated as described herein) to a mammalian cell can be used to silence expression of a target gene while circumventing the interferon response. Further, use of a discrete species of siRNA can be used to selectively target one allele of a target gene, *e.g.*, in a subject heterozygous for the allele.

Moreover, in one embodiment, a mammalian cell is treated with siRNAs that disrupt a component of the interferon response, *e.g.*, double stranded RNA (dsRNA)-activated protein kinase PKR. Such a cell can be treated with a second set of dsRNAs that include a sequence complementary to a target RNA and that have a length that might otherwise trigger the interferon response.

In a typical embodiment, the subject is a mammal such as a cow, horse, mouse, rat, dog, pig, goat, primate. In a preferred embodiment, the subject is a dairy mammal (*e.g.*, a cow, or goat) or other farmed animal (*e.g.*, a chicken, turkey, sheep, pig, fish, shrimp). In a much preferred embodiment, the subject is a human, *e.g.*, a normal individual or an individual that has, is diagnosed with, or is predicted to have a disease or disorder.

Further, because siRNA mediated silencing persists for several days after administering the siRNA composition, in many instances, it is possible to administer the composition with a frequency of less than once per day, or, for some instances, only once for the entire therapeutic regimen. For example, treatment of some cancer cells may be mediated by a single bolus administration, whereas a chronic viral infection may require regular administration, *e.g.*, once per week or once per month.

A number of exemplary routes of delivery are described that can be used to administer an siRNAs to a subject. In addition, the siRNAs can be formulated according to an exemplary method described herein.

The modulation of gene expression by one or more siRNAs can be evaluated by methods that measure, *e.g.*, the level of transcription of the target gene, the level of protein encoded by the target gene, covalent modifications of the target gene or of proteins associated with the target gene, *e.g.*, methylation or acetylation, or the ability of the genomic locus encoding the target gene to be transcribed.

Alterations in the level of transcription can be determined, for example, by measuring the abundance of mRNA transcripts of the target gene. The level of mRNA in a sample that is encoded by the target gene can be evaluated with nucleic acid amplification, *e.g.*, by RT-PCR (Mullis (1987) U.S. Patent No. 4,683,202), ligase chain reaction (Barany (1991) Proc. Natl. Acad. Sci. USA 88:189-193), self sustained sequence replication (Guatelli *et al.*, (1990) Proc. Natl. Acad. Sci. USA 87:1874-1878), transcriptional amplification system (Kwoh *et al.*, (1989), Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi *et al.*, (1988) Bio/Technology 6:1197), rolling circle replication (Lizardi *et al.*, U.S. Patent No. 5,854,033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques known in the art. As used herein, amplification primers are defined as being a pair of nucleic acid molecules that can anneal to 5' or 3' regions of a gene (plus and minus strands, respectively, or vice-versa) and contain a short region in between. In general, amplification primers are from about 10 to 30 nucleotides in length and flank a region from about 50 to 200 nucleotides in length. Under appropriate conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule comprising the nucleotide sequence flanked by the primers.

For *in situ* methods, a cell or tissue sample can be prepared/processed and immobilized on a support, typically a glass slide, and then contacted with a probe that can hybridize to mRNA that encodes the target gene being analyzed.

A variety of methods can be used to determine the level of protein encoded by the target gene. In general, these methods include contacting an agent that selectively binds to the protein, such as an antibody with a sample, to evaluate the level of protein in the sample. In a preferred embodiment, the antibody bears a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (*e.g.*, Fab or F(ab')2) can be used. The term "labeled," with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with a detectable substance.

The detection methods can be used to detect protein encoded by the target gene in a biological sample *in vitro* as well as *in vivo*. *In vitro* techniques for detection protein include enzyme linked immunosorbent assays (ELISAs), immunoprecipitations, immunofluorescence, enzyme immunoassay (EIA), radioimmunoassay (RIA), and Western blot analysis. *In vivo* techniques for detection of protein include introducing into a subject a labeled antibody, *e.g.*, a biotinylated antibody or an antibody labeled with a radioactive marker. Antibody-based detection methods can also be used to detect covalent modifications such as ubiquitination and phosphorylation.

Administration of RNA can cause modification of proteins associated with target genes, *e.g.*, chromosomal proteins, *e.g.*, histones. Chromosomal proteins can be subject to numerous types of chemical modification, many or all of which can alter chromatin structure, thereby altering the ability of genes encoded in the particular chromosomal region to be transcribed. For example, histones are subject to acetylation by histone acetyltransferases, deacetylation by histone deacetylases, methylation by histone methyltransferases (and therefore presumably to demethylation by histone demethylases), ubiquitination by ubiquitin ligases, de-ubiquitination by ubiquitin hydrolases, phosphorylation by histone kinases, dephosphorylation by histone phosphatases, and reversible ADP-ribosylation by poly-

ADP ribose polymerase (PARP, also known as TFIIC) (Strahl *et al.* (2000) Nature 403:41-45). For review, see, *e.g.*, Geiman TM, Robertson KD. J Cell Biochem. 2002;87(2):117-25. Techniques useful for detecting methylation include, *e.g.*, those described by Ahrendt *et al.* (1999), Belinsky *et al.* (1998), Clark *et al.* (1994), Herman *et al.* (1996) and Xiong, Z. and Laird, P. W. (1997). Techniques for determining the accessibility of a region of chromatin to transcription factors include methods that detect its sensitivity to chemical and enzymatic probes, for example, nucleases.

siRNA PRODUCTION

An siRNA can be produced, *e.g.*, in bulk, by a variety of methods. Exemplary methods include: organic synthesis and dsRNA cleavage, *e.g.*, *in vitro* cleavage.

Organic Synthesis

An siRNA can be made by separately synthesizing each respective strand of the double-stranded RNA molecule. The component strands can then be annealed.

A large bioreactor, *e.g.*, the OligoPilot II from Pharmacia Biotec AB (Uppsala Sweden), can be used to produce a large amount of a particular RNA strand for a given siRNA. The OligoPilotII reactor can efficiently couple a nucleotide using only a 1.5 molar excess of a phosphoramidite nucleotide. To make an RNA strand, ribonucleotides amidites are used. Standard cycles of monomer addition can be used to synthesize the 21 to 23 nucleotide strand for the siRNA. Typically, the two complementary strands are produced separately and then annealed, *e.g.*, after release from the solid support and deprotection.

Organic synthesis can be used to produce a discrete siRNA species. The complementary of the species to a particular target gene can be precisely specified. For example, the species may be complementary to a region that includes a polymorphism, *e.g.*, a single nucleotide polymorphism. Further the location of the polymorphism can be precisely defined. In some embodiments, the polymorphism is located in an internal region, *e.g.*, at least 4, 5, 7, or 9 nucleotides from one or both of the termini.

RNAs may be used which contain phosphorothioate groups to enhance stability. See, *e.g.*, EP1224193, US6403781, Klein JM, *et al.*, *Am J Respir Cell Mol Biol.* 2000 Jun;22(6):676-84.

dsRNA Cleavage

siRNAs can also be made by cleaving a larger dsRNA. The cleavage can be mediated *in vitro* or *in vivo*. For example, to produce siRNAs by cleavage *in vitro*, the following method can be used:

In vitro transcription. dsRNA is produced by transcribing a nucleic acid (DNA) segment in both directions. For example, the HiScribe™ RNAi transcription kit (New England Biolabs) provides a vector and a method for producing a dsRNA for a nucleic acid segment that is cloned into the vector at a position flanked on either side by a T7 promoter. Separate templates are generated for T7 transcription of the two complementary strands for the dsRNA. The templates are transcribed *in vitro* by addition of T7 RNA polymerase and dsRNA is produced. Similar methods using PCR and/or other RNA polymerases (*e.g.*, T3 or SP6 polymerase) can also be used. In one embodiment, RNA generated by this method is carefully purified to remove endotoxins that may contaminate preparations of the recombinant enzymes.

In vitro cleavage. dsRNA is cleaved *in vitro* into siRNAs, for example, using a Dicer or comparable RNAse III-based activity. For example, the dsRNA can be incubated in an *in vitro* extract from Drosophila or using purified components, *e.g.* a purified RNAse or RISC complex (RNA-induced silencing complex). See, *e.g.*, Ketting *et al. Genes Dev* 2001 Oct 15;15(20):2654-9. and Hammond *Science* 2001 Aug 10;293(5532):1146-50.

dsRNA cleavage generally produces a plurality of siRNA species, each being a particular 21 to 23 nt fragment of a source dsRNA molecule. For example, siRNAs that include sequences complementary to overlapping regions and adjacent regions of a source dsRNA molecule may be present.

Regardless of the method of synthesis, the siRNA preparation can be prepared in a solution (*e.g.*, an aqueous and/or organic solution) that is appropriate for formulation. For example, the siRNA preparation can be precipitated and redissolved in pure double-distilled water, and lyophilized. The dried siRNA can then be resuspended in a solution appropriate for the intended formulation process.

FORMULATION

A formulated siRNA composition can assume a variety of states. In some examples, the composition is at least partially crystalline, uniformly crystalline, and/or anhydrous (*e.g.*, less than 80, 50, 30, 20, or 10% water). In another example, the siRNA is in an aqueous phase, *e.g.*, in a solution that includes water.

The aqueous phase or the crystalline compositions can, *e.g.*, be incorporated into a delivery vehicle, *e.g.*, a liposome (particularly for the aqueous phase) or a particle (*e.g.*, a microparticle as can be appropriate for a crystalline composition). Generally, the siRNA composition is formulated in a manner that is compatible with the intended method of administration (see, below).

In particular embodiments; the composition is prepared by at least one of the following methods: spray drying, lyophilization, vacuum drying, evaporation, fluid bed drying, or a combination of these techniques; or sonication with a lipid, freeze-drying, condensation and other self-assembly.

An siRNA preparation can be formulated in combination with another agent, *e.g.*, another therapeutic agent or an agent that stabilizes an siRNA, *e.g.*, a protein that complexes with siRNA to form an siRNP. Still other agents include chelators, *e.g.*, EDTA (*e.g.*, to remove divalent cations such as Mg^{2+}), salts, RNAse inhibitors (*e.g.*, a broad specificity RNAse inhibitor such as RNAsin) and so forth.

In one embodiment, the siRNA preparation includes another set of siRNA agents, *e.g.*, a second set of siRNAs that can mediated RNAi with respect to a second gene, or with respect to the same gene. Still other preparation can include at least 3, 5, ten, twenty, fifty, or a hundred or more different siRNA species. Such siRNAs can mediated RNAi with respect to a similar number of different genes.

In one embodiment, the siRNA preparation includes at least a second therapeutic agent (*e.g.*, an agent other than an RNA or a DNA). For example, an siRNA composition for the treatment of a viral disease, *e.g.* HIV, might include a known antiviral agent (*e.g.*, a protease inhibitor or reverse transcriptase inhibitor). In another example, an siRNA composition for the treatment of a cancer might further comprise a chemotherapeutic agent.

Exemplary formulations are discussed below:

Liposomes

An siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) preparation can be formulated for delivery in a membranous molecular assembly, *e.g.*, a liposome or a micelle. As used herein, the term "liposome" refers to a vesicle composed of amphiphilic lipids arranged in at least one bilayer, *e.g.*, one bilayer or a plurality of bilayers. Liposomes include unilamellar and multilamellar vesicles that have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the siRNA composition. The lipophilic material isolates the aqueous interior from an aqueous exterior, which typically does not include the siRNA composition, although in some examples, it may. Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomal bilayer fuses with bilayer of the cellular membranes. As the merging of the liposome and cell progresses, the internal aqueous contents that include the siRNA are delivered into the cell where the siRNA can specifically bind to a target RNA and can mediate RNAi. In some cases the liposomes are also specifically targeted, *e.g.*, to direct the siRNA to particular cell types.

A liposome containing an siRNA can be prepared by a variety of methods.

In one example, the lipid component of a liposome is dissolved in a detergent so that micelles are formed with the lipid component. For example, the lipid component can be an amphipathic cationic lipid or lipid conjugate. The detergent can have a high critical micelle concentration and may be nonionic. Exemplary detergents include cholate, CHAPS, octylglucoside, deoxycholate, and lauroyl sarcosine. The siRNA preparation is then added to the micelles that include the lipid component. The cationic groups on the lipid interact with the siRNA and condense around the siRNA to form a liposome. After condensation, the detergent is removed, *e.g.*, by dialysis, to yield a liposomal preparation of siRNA.

If necessary a carrier compound that assists in condensation can be added during the condensation reaction, *e.g.*, by controlled addition. For example, the

carrier compound can be a polymer other than a nucleic acid (*e.g.*, spermine or spermidine). pH can also adjusted to favor condensation.

Further description of methods for producing stable polynucleotide delivery vehicles, which incorporate a polynucleotide/cationic lipid complex as structural components of the delivery vehicle, are described in, *e.g.*, WO 96/37194. Liposome formation can also include one or more aspects of exemplary methods described in Felgner, P. L. *et al.*, *Proc. Natl. Acad. Sci.*, USA 8:7413-7417; 1987; U.S. Pat. No. 4,897,355; U.S. Pat. No. 5,171,678; Bangham, *et al. M. Mol. Biol.* 23:238, 1965; Olson, *et al. Biochim. Biophys. Acta* 557:9, 1979; Szoka, *et al. Proc. Natl. Acad. Sci.* 75: 4194, 1978; Mayhew, *et al. Biochim. Biophys. Acta* 775:169, 1984; Kim, *et al. Biochim. Biophys. Acta* 728:339, 1983; and Fukunaga, *et al. Endocrinol.* 115:757, 1984. Commonly used techniques for preparing lipid aggregates of appropriate size for use as delivery vehicles include sonication and freeze-thaw plus extrusion (see, *e.g.*, Mayer, *et al. Biochim. Biophys. Acta* 858:161, 1986). Microfluidization can be used when consistently small (50 to 200 nm) and relatively uniform aggregates are desired (Mayhew, *et al. Biochim. Biophys. Acta* 775:169, 1984). These methods are readily adapted to packaging siRNA preparations into liposomes.

Liposomes that are pH-sensitive or negatively-charged, entrap nucleic acid molecules rather than complex with them. Since both the nucleic acid molecules and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some nucleic acid molecules are entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, *Journal of Controlled Release*, 19, (1992) 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example,

soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Examples of other methods to introduce liposomes into cells *in vitro* and *in vivo* include U.S. Pat. No. 5,283,185; U.S. Pat. No. 5,171,678; WO 94/00569; WO 93/24640; WO 91/16024; Felgner, *J. Biol. Chem.* 269:2550, 1994; Nabel, *Proc. Natl. Acad. Sci.* 90:11307, 1993; Nabel, *Human Gene Ther.* 3:649, 1992; Gershon, *Biochem.* 32:7143, 1993; and Strauss *EMBO J.* 11:417, 1992.

In one embodiment, cationic liposomes are used. Cationic liposomes possess the advantage of being able to fuse to the cell membrane. Non-cationic liposomes, although not able to fuse as efficiently with the plasma membrane, are taken up by macrophages *in vivo* and can be used to deliver siRNAs to macrophages.

Further advantages of liposomes include: liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated siRNAs in their internal compartments from metabolism and degradation (Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

A positively charged synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) can be used to form small liposomes that interact spontaneously with nucleic acid to form lipid-nucleic acid complexes which are capable of fusing with the negatively charged lipids of the cell membranes of tissue culture cells, resulting in delivery of siRNA (see, *e.g.*, Felgner, P. L. *et al.*, Proc. Natl. Acad. Sci., USA 8:7413-7417, 1987 and U.S. Pat. No. 4,897,355 for a description of DOTMA and its use with DNA).

A DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonia)propane (DOTAP) can be used in combination with a phospholipid to form DNA-complexing vesicles. Lipofectin™ Bethesda Research Laboratories, Gaithersburg, Md.) is an effective agent for the delivery of highly anionic nucleic acids into living tissue culture cells that comprise positively charged DOTMA liposomes which interact spontaneously with negatively charged polynucleotides to form complexes. When enough positively charged liposomes are used, the net charge on the resulting

complexes is also positive. Positively charged complexes prepared in this way spontaneously attach to negatively charged cell surfaces, fuse with the plasma membrane, and efficiently deliver functional nucleic acids into, for example, tissue culture cells. Another commercially available cationic lipid, 1,2-bis(oleoyloxy)-3,3-(trimethylammonia)propane ("DOTAP") (Boehringer Mannheim, Indianapolis, Indiana) differs from DOTMA in that the oleoyl moieties are linked by ester, rather than ether linkages.

Other reported cationic lipid compounds include those that have been conjugated to a variety of moieties including, for example, carboxyspermine which has been conjugated to one of two types of lipids and includes compounds such as 5-carboxyspermylglycine dioctaoleoylamide ("DOGS") (Transfectam™, Promega, Madison, Wisconsin) and dipalmitoylphosphatidylethanolamine 5-carboxyspermyl-amide ("DPPES") (see, *e.g.*, U.S. Pat. No. 5,171,678).

Another cationic lipid conjugate includes derivatization of the lipid with cholesterol ("DC-Chol") which has been formulated into liposomes in combination with DOPE (See, Gao, X. and Huang, L., Biochim. Biophys. Res. Commun. 179:280, 1991). Lipopolylysine, made by conjugating polylysine to DOPE, has been reported to be effective for transfection in the presence of serum (Zhou, X. *et al.*, Biochim. Biophys. Acta 1065:8, 1991). For certain cell lines, these liposomes containing conjugated cationic lipids, are said to exhibit lower toxicity and provide more efficient transfection than the DOTMA-containing compositions. Other commercially available cationic lipid products include DMRIE and DMRIE-HP (Vical, La Jolla, California) and Lipofectamine (DOSPA) (Life Technology, Inc., Gaithersburg, Maryland). Other cationic lipids suitable for the delivery of oligonucleotides are described in WO 98/39359 and WO 96/37194.

Liposomal formulations are particularly suited for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer siRNA, into the skin. In some implementations, liposomes are used for delivering siRNA to epidermal cells and also to enhance the penetration of siRNA into dermal tissues, *e.g.*, into skin. For example, the liposomes can be applied

topically. Topical delivery of drugs formulated as liposomes to the skin has been documented (see, *e.g.*, Weiner *et al.*, *Journal of Drug Targeting*, 1992, vol. 2,405-410 and du Plessis *et al.*, *Antiviral Research*, 18, 1992, 259-265; Mannino, R. J. and Fould-Fogerite, S., Biotechniques 6:682-690, 1988; Itani, T. *et al.* Gene 56:267-276. 1987; Nicolau, C. *et al.* Meth. Enz. 149:157-176, 1987; Straubinger, R. M. and Papahadjopoulos, D. Meth. Enz. 101:512-527, 1983; Wang, C. Y. and Huang, L., Proc. Natl. Acad. Sci. USA 84:7851-7855, 1987).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome II (glyceryl distearate/ cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver a drug into the dermis of mouse skin. Such formulations with siRNA are useful for treating a dermatological disorder.

Liposomes that include siRNA can be made highly deformable. Such deformability can enable the liposomes to penetrate through pore that are smaller than the average radius of the liposome. For example, transfersomes are a type of deformable liposomes. Transferosomes can be made by adding surface edge activators, usually surfactants, to a standard liposomal composition. Transfersomes that include siRNA can be delivered, for example, subcutaneously by infection in order to deliver siRNA to keratinocytes in the skin. In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. In addition, due to the lipid properties, these transferosomes can be self-optimizing (adaptive to the shape of pores, *e.g.*, in the skin), self-repairing, and can frequently reach their targets without fragmenting, and often self-loading.

Surfactants

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes (see above). siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) compositions can include a surfactant. In one embodiment, the siRNA

is formulated as an emulsion that includes a surfactant. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in "Pharmaceutical Dosage Forms," Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in "Pharmaceutical Dosage Forms," Marcel Dekker, Inc., New York, NY, 1988, p. 285).

Micelles and other Membranous Formulations

The siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) composition can be provided as a micellar formulation. "Micelles" are defined herein as a particular type of molecular assembly in which amphipathic molecules are arranged in a spherical structure such that all the hydrophobic portions of the molecules are directed inward, leaving the hydrophilic portions in contact with the surrounding aqueous phase. The converse arrangement exists if the environment is hydrophobic.

A mixed micellar formulation suitable for delivery through transdermal membranes may be prepared by mixing an aqueous solution of the siRNA composition, an alkali metal C_8 to C_{22} alkyl sulphate, and a micelle forming compounds. Exemplary micelle forming compounds include lecithin, hyaluronic acid, pharmaceutically acceptable salts of hyaluronic acid, glycolic acid, lactic acid, chamomile extract, cucumber extract, oleic acid, linoleic acid, linolenic acid, monoolein, monooleates, monolaurates, borage oil, evening of primrose oil, menthol, trihydroxy oxo cholanyl glycine and pharmaceutically acceptable salts thereof, glycerin, polyglycerin, lysine, polylysine, triolein, polyoxyethylene ethers and analogues thereof, polidocanol alkyl ethers and analogues thereof, chenodeoxycholate, deoxycholate, and mixtures thereof. The micelle forming compounds may be added at the same time or after addition of the alkali metal alkyl sulphate. Mixed micelles will form with substantially any kind of mixing of the ingredients but vigorous mixing is preferred in order to provide smaller size micelles.

In one method a first micellar composition is prepared which contains the siRNA composition and at least the alkali metal alkyl sulphate. The first micellar composition is then mixed with at least three micelle forming compounds to form a mixed micellar composition. In another method, the micellar composition is prepared by mixing the siRNA composition, the alkali metal alkyl sulphate and at least one of

the micelle forming compounds, followed by addition of the remaining micelle forming compounds, with vigorous mixing.

Phenol and/or m-cresol may be added to the mixed micellar composition to stabilize the formulation and protect against bacterial growth. Alternatively, phenol and/or m-cresol may be added with the micelle forming ingredients. An isotonic agent such as glycerin may also be added after formation of the mixed micellar composition.

For delivery of the micellar formulation as a spray, the formulation can be put into an aerosol dispenser and the dispenser is charged with a propellant. The propellant, which is under pressure, is in liquid form in the dispenser. The ratios of the ingredients are adjusted so that the aqueous and propellant phases become one, i.e. there is one phase. If there are two phases, it is necessary to shake the dispenser prior to dispensing a portion of the contents, *e.g.* through a metered valve. The dispensed dose of pharmaceutical agent is propelled from the metered valve in a fine spray.

The preferred propellants are hydrogen-containing chlorofluorocarbons, hydrogen-containing fluorocarbons, dimethyl ether and diethyl ether. Even more preferred is HFA 134a (1,1,1,2 tetrafluoroethane).

The specific concentrations of the essential ingredients can be determined by relatively straightforward experimentation. For absorption through the oral cavities, it is often desirable to increase, *e.g.* at least double or triple, the dosage for through injection or administration through the gastrointestinal tract.

Particles

In another embodiment, siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) preparations may be incorporated into a particle, *e.g.*, a microparticle. Microparticles can be produced by spray-drying, but may also be produced by other methods including lyophilization, evaporation, fluid bed drying, vacuum drying, or a combination of these techniques. See below for further description.

Sustained -Release Formulations. siRNAs (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) described herein can be formulated for controlled, *e.g.*, slow release.

Controlled release can be achieved by disposing the siRNA within a structure or substance that impedes its release. *E.g.*, siRNA can be disposed within a porous matrix or in an erodable matrix, either of which allow release of the siRNA over a period of time.

Polymeric particles, *e.g.*, polymeric in microparticles can be used as a sustained-release reservoir of siRNA that is taken up by cells only released from the microparticle through biodegradation. The polymeric particles in this embodiment should therefore be large enough to preclude phagocytosis (*e.g.*, larger than 10 μm and preferably larger than 20 μm). Such particles can be produced by the same methods to make smaller particles, but with less vigorous mixing of the first and second emulsions. That is to say, a lower homogenization speed, vortex mixing speed, or sonication setting can be used to obtain particles having a diameter around 100 μm rather than 10 μm. The time of mixing also can be altered.

Larger microparticles can be formulated as a suspension, a powder, or an implantable solid, to be delivered by intramuscular, subcutaneous, intradermal, intravenous, or intraperitoneal injection; via inhalation (intranasal or intrapulmonary); orally; or by implantation. These particles are useful for delivery of any siRNA when slow release over a relatively long term is desired. The rate of degradation, and consequently of release, varies with the polymeric formulation.

Microparticles preferably include pores, voids, hollows, defects or other interstitial spaces that allow the fluid suspension medium to freely permeate or perfuse the particulate boundary. For example, the perforated microstructures can be used to form hollow, porous spray dried microspheres.

Polymeric particles containing siRNA (or other dsRNAs) can be made using a double emulsion technique, for instance. First, the polymer is dissolved in an organic solvent. A preferred polymer is polylactic-co-glycolic acid (PLGA), with a lactic/glycolic acid weight ratio of 65:35, 50:50, or 75:25. Next, a sample of nucleic acid suspended in aqueous solution is added to the polymer solution and the two solutions are mixed to form a first emulsion. The solutions can be mixed by vortexing or shaking, and in a preferred method, the mixture can be sonicated. Most preferable is any method by which the nucleic acid receives the least amount of damage in the form of nicking, shearing, or degradation, while still allowing the formation of an

appropriate emulsion. For example, acceptable results can be obtained with a Vibra-cell model VC-250 sonicator with a 1/8" microtip probe, at setting #3.

Spray-Drying

siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) can be prepared by spray drying. Spray dried siRNA can be administered to a subject or be subjected to further formulation. A pharmaceutical composition of siRNA can be prepared by spray drying a homogeneous aqueous mixture that includes an siRNA under conditions sufficient to provide a dispersible powdered composition, *e.g.*, a pharmaceutical composition. The material for spray drying can also include one or more of: a pharmaceutically acceptable excipient, or a dispersibility-enhancing amount of a physiologically acceptable, water-soluble protein. The spray-dried product can be a dispersible powder that includes the siRNA.

Spray drying is a process that converts a liquid or slurry material to a dried particulate form. Spray drying can be used to provide powdered material for various administrative routes including inhalation. See, for example, M. Sacchetti and M. M. Van Oort in: Inhalation Aerosols: Physical and Biological Basis for Therapy, A. J. Hickey, ed. Marcel Dekkar, New York, 1996.

Spray drying can include atomizing a solution, emulsion, or suspension to form a fine mist of droplets and drying the droplets. The mist can be projected into a drying chamber (*e.g.*, a vessel, tank, tubing, or coil) where it contacts a drying gas. The mist can include solid or liquid pore forming agents. The solvent and pore forming agents evaporate from the droplets into the drying gas to solidify the droplets, simultaneously forming pores throughout the solid. The solid (typically in a powder, particulate form) then is separated from the drying gas and collected.

Spray drying includes bringing together a highly dispersed liquid, and a sufficient volume of air (*e.g.*, hot air) to produce evaporation and drying of the liquid droplets. The preparation to be spray dried can be any solution, course suspension, slurry, colloidal dispersion, or paste that may be atomized using the selected spray drying apparatus. Typically, the feed is sprayed into a current of warm filtered air that evaporates the solvent and conveys the dried product to a collector. The spent air is

then exhausted with the solvent. Several different types of apparatus may be used to provide the desired product. For example, commercial spray dryers manufactured by Buchi Ltd. or Niro Corp. can effectively produce particles of desired size.

Spray-dried powdered particles can be approximately spherical in shape, nearly uniform in size and frequently hollow. There may be some degree of irregularity in shape depending upon the incorporated medicament and the spray drying conditions. In many instances the dispersion stability of spray-dried microspheres appears to be more effective if an inflating agent (or blowing agent) is used in their production. Particularly preferred embodiments may comprise an emulsion with an inflating agent as the disperse or continuous phase (the other phase being aqueous in nature). An inflating agent is preferably dispersed with a surfactant solution, using, for instance, a commercially available microfluidizer at a pressure of about 5000 to 15,000 psi. This process forms an emulsion, preferably stabilized by an incorporated surfactant, typically comprising submicron droplets of water immiscible blowing agent dispersed in an aqueous continuous phase. The formation of such dispersions using this and other techniques are common and well known to those in the art. The blowing agent is preferably a fluorinated compound (*e.g.* perfluorohexane, perfluorooctyl bromide, perfluorodecalin, perfluorobutyl ethane) that vaporizes during the spray-drying process, leaving behind generally hollow, porous aerodynamically light microspheres. As will be discussed in more detail below, other suitable blowing agents include chloroform, freons, and hydrocarbons. Nitrogen gas and carbon dioxide are also contemplated as a suitable blowing agent.

Although the perforated microstructures are preferably formed using a blowing agent as described above, it will be appreciated that, in some instances, no blowing agent is required and an aqueous dispersion of the medicament and surfactant(s) are spray dried directly. In such cases, the formulation may be amenable to process conditions (*e.g.*, elevated temperatures) that generally lead to the formation of hollow, relatively porous microparticles. Moreover, the medicament may possess special physicochemical properties (*e.g.*, high crystallinity, elevated melting temperature, surface activity, etc.) that make it particularly suitable for use in such techniques.

The perforated microstructures may optionally be associated with, or comprise, one or more surfactants. Moreover, miscible surfactants may optionally be combined with the suspension medium liquid phase. It will be appreciated by those skilled in the art that the use of surfactants may further increase dispersion stability, simplify formulation procedures or increase bioavailability upon administration. Of course combinations of surfactants, including the use of one or more in the liquid phase and one or more associated with the perforated microstructures are contemplated as being within the scope of the invention. By "associated with or comprise" it is meant that the structural matrix or perforated microstructure may incorporate, adsorb, absorb, be coated with or be formed by the surfactant.

Surfactants suitable for use include any compound or composition that aids in the formation and maintenance of the stabilized respiratory dispersions by forming a layer at the interface between the structural matrix and the suspension medium. The surfactant may comprise a single compound or any combination of compounds, such as in the case of co-surfactants. Particularly preferred surfactants are substantially insoluble in the propellant, nonfluorinated, and selected from the group consisting of saturated and unsaturated lipids, nonionic detergents, nonionic block copolymers, ionic surfactants, and combinations of such agents. It should be emphasized that, in addition to the aforementioned surfactants, suitable (i.e. biocompatible) fluorinated surfactants are compatible with the teachings herein and may be used to provide the desired stabilized preparations.

Lipids, including phospholipids, from both natural and synthetic sources may be used in varying concentrations to form a structural matrix. Generally, compatible lipids comprise those that have a gel to liquid crystal phase transition greater than about 40° C. Preferably, the incorporated lipids are relatively long chain (i.e. C_6 -C_{22}) saturated lipids and more preferably comprise phospholipids. Exemplary phospholipids useful in the disclosed stabilized preparations comprise egg phosphatidylcholine, dilauroylphosphatidylcholine, dioleylphosphatidylcholine, dipalmitoylphosphatidyl-choline, disteroylphosphatidylcholine, short-chain phosphatidylcholines, phosphatidylethanolamine, dioleylphosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, glycolipids, ganglioside GM1, sphingomyelin, phosphatidic acid, cardiolipin; lipids bearing

polymer chains such as, polyethylene glycol, chitin, hyaluronic acid, or polyvinylpyrrolidone; lipids bearing sulfonated mono-, di-, and polysaccharides; fatty acids such as palmitic acid, stearic acid, and oleic acid; cholesterol, cholesterol esters, and cholesterol hemisuccinate. Due to their excellent biocompatibility characteristics, phospholipids and combinations of phospholipids and poloxamers are particularly suitable for use in the stabilized dispersions disclosed herein.

Compatible nonionic detergents comprise: sorbitan esters including sorbitan trioleate (Spans™ 85), sorbitan sesquioleate, sorbitan monooleate, sorbitan monolaurate, polyoxyethylene (20) sorbitan monolaurate, and polyoxyethylene (20) sorbitan monooleate, oleyl polyoxyethylene (2) ether, stearyl polyoxyethylene (2) ether, lauryl polyoxyethylene (4) ether, glycerol esters, and sucrose esters. Other suitable nonionic detergents can be easily identified using McCutcheon's Emulsifiers and Detergents (McPublishing Co., Glen Rock, N.J.). Preferred block copolymers include diblock and triblock copolymers of polyoxyethylene and polyoxypropylene, including poloxamer 188 (Pluronic.RTM. F68), poloxamer 407 (Pluronic.RTM. F-127), and poloxamer 338. Ionic surfactants such as sodium sulfosuccinate, and fatty acid soaps may also be utilized. In preferred embodiments, the microstructures may comprise oleic acid or its alkali salt.

In addition to the aforementioned surfactants, cationic surfactants or lipids are preferred especially in the case of delivery of dsRNA, *e.g.*, an siRNA. Examples of suitable cationic lipids include: DOTMA, N-[-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium-chloride; DOTAP,1,2-dioleyloxy-3-(trimethylammonio)propane; and DOTB, 1,2-dioleyl-3-(4'-trimethylammonio)butanoyl-sn-glycerol. Polycationic amino acids such as polylysine, and polyarginine are also contemplated.

For the spraying process, such spraying methods as rotary atomization, pressure atomization and two-fluid atomization can be used. Examples of the devices used in these processes include "Parubisu [phonetic rendering] Mini-Spray GA-32" and "Parubisu Spray Drier DL-41", manufactured by Yamato Chemical Co., or "Spray Drier CL-8," "Spray Drier L-8," "Spray Drier FL-12," "Spray Drier FL-16" or "Spray Drier FL-20," manufactured by Okawara Kakoki Co., can be used for the method of spraying using rotary-disk atomizer.

While no particular restrictions are placed on the gas used to dry the sprayed material, it is recommended to use air, nitrogen gas or an inert gas. The temperature of the inlet of the gas used to dry the sprayed materials such that it does not cause heat deactivation of the sprayed material. The range of temperatures may vary between about 50°C to about 200°C, preferably between about 50°C and 100°C. The temperature of the outlet gas used to dry the sprayed material, may vary between about 0°C and about 150°C, preferably between 0°C and 90°C, and even more preferably between 0°C and 60°C.

The spray drying is done under conditions that result in substantially amorphous powder of homogeneous constitution having a particle size that is respirable, a low moisture content and flow characteristics that allow for ready aerosolization. Preferably the particle size of the resulting powder is such that more than about 98% of the mass is in particles having a diameter of about 10 μm or less with about 90% of the mass being in particles having a diameter less than 5 μm. Alternatively, about 95% of the mass will have particles with a diameter of less than 10 μm with about 80% of the mass of the particles having a diameter of less than 5 μm.

The dispersible pharmaceutical-based dry powders that include the siRNA preparation may optionally be combined with pharmaceutical carriers or excipients that are suitable for respiratory and pulmonary administration. Such carriers may serve simply as bulking agents when it is desired to reduce the siRNA concentration in the powder which is being delivered to a patient, but may also serve to enhance the stability of the siRNA compositions and to improve the dispersibility of the powder within a powder dispersion device in order to provide more efficient and reproducible delivery of the siRNA and to improve handling characteristics of the siRNA such as flowability and consistency to facilitate manufacturing and powder filling.

Such carrier materials may be combined with the drug prior to spray drying, i.e., by adding the carrier material to the purified bulk solution. In that way, the carrier particles will be formed simultaneously with the drug particles to produce a homogeneous powder. Alternatively, the carriers may be separately prepared in a dry powder form and combined with the dry powder drug by blending. The powder carriers will usually be crystalline (to avoid water absorption), but might in some

cases be amorphous or mixtures of crystalline and amorphous. The size of the carrier particles may be selected to improve the flowability of the drug powder, typically being in the range from 25 μm to 100 μm. A preferred carrier material is crystalline lactose having a size in the above-stated range.

Powders prepared by any of the above methods will be collected from the spray dryer in a conventional manner for subsequent use. For use as pharmaceuticals and other purposes, it will frequently be desirable to disrupt any agglomerates that may have formed by screening or other conventional techniques. For pharmaceutical uses, the dry powder formulations will usually be measured into a single dose, and the single dose sealed into a package. Such packages are particularly useful for dispersion in dry powder inhalers, as described in detail below. Alternatively, the powders may be packaged in multiple-dose containers.

Methods for spray drying hydrophobic and other drugs and components are described in U.S. Pat. Nos. 5,000,888; 5,026,550; 4,670,419, 4,540,602; and 4,486,435. Bloch and Speison (1983) Pharm. Acta Helv 58:14-22 teaches spray drying of hydrochlorothiazide and chlorthalidone (lipophilic drugs) and a hydrophilic adjuvant (pentaerythritol) in azeotropic solvents of dioxane-water and 2-ethoxyethanol-water. A number of Japanese Patent application Abstracts relate to spray drying of hydrophilic-hydrophobic product combinations, including JP 806766; JP 7242568; JP 7101884; JP 7101883; JP 71018982; JP 7101881; and JP 4036233. Other foreign patent publications relevant to spray drying hydrophilic-hydrophobic product combinations include FR 2594693; DE 2209477; and WO 88/07870.

Lyophilization

siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) preparations can be made by lyophilization. Lyophilization is a freeze-drying process in which water is sublimed from the composition after it is frozen. The particular advantage associated with the lyophilization process is that biologicals and pharmaceuticals that are relatively unstable in an aqueous solution can be dried without elevated temperatures (thereby eliminating the adverse thermal effects), and then stored in a dry state where there are few stability problems. With respect to the instant invention such techniques

are particularly compatible with the incorporation of nucleic acids in perforated microstructures without compromising physiological activity. Methods for providing lyophilized particulates are known to those of skill in the art and it would clearly not require undue experimentation to provide dispersion compatible microstructures in accordance with the teachings herein. Accordingly, to the extent that lyophilization processes may be used to provide microstructures having the desired porosity and size, they are conformance with the teachings herein and are expressly contemplated as being within the scope of the instant invention.

Viral Encapsulation

siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) can be bound to, associated with or surrounded by, at least one viral coat protein that originates from a virus, is derived therefrom or has been prepared synthetically. The coat protein can be derived from polyomavirus, and can contain the polyomavirus protein 1 (VP1) and/or virus protein 2 (VP2). The use of such coat proteins is referenced in DE 19618797 A1, for instance. When a capsid or capsid-type structure is formed from the coat protein, one side preferably faces the interior of the capsid or capsid-type structure. This structure is particularly stable.

Targeting

In some embodiments, one or more siRNAs (or precursors, including, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) is targeted to a particular cell. For example, a liposome or particle or other structure that includes an siRNA can also include a targeting moiety that recognizes a specific molecule on a target cell. The targeting moiety can be a molecule with a specific affinity for a target cell. Targeting moieties can include antibodies directed against a protein found on the surface of a target cell, or the ligand or a receptor-binding portion of a ligand for a molecule found on the surface of a target cell. For example, the targeting moiety can recognize a cancer-specific antigen (*e.g.*, CA15-3, CA19-9, CEA, or HER2/neu.) or a viral antigen, thus delivering the siRNA to a cancer cell or a virus-infected cell. Exemplary targeting moieties include

antibodies (such as IgM, IgG, IgA, IgD, and the like, or a functional portions thereof), ligands for cell surface receptors (*e.g.*, ectodomains thereof).

Table 1 provides a number of antigens that can be used to target selected cells.

Table 1. Antigens found in tumor tissues.

ANTIGEN	Exemplary tumor tissue
CEA (carcinoembryonic antigen)	colon, breast, lung
PSA (prostate specific antigen)	prostate cancer
CA-125	ovarian cancer
CA 15-3	breast cancer
CA 19-9	breast cancer
HER2/neu	breast cancer
α-feto protein	testicular cancer, hepatic cancer
β-HCG (human chorionic gonadotropin)	testicular cancer, choriocarcinoma
MUC-1	breast cancer
Estrogen receptor	breast cancer, uterine cancer
Progesterone receptor	breast cancer, uterine cancer
EGFr (epidermal growth factor receptor)	bladder cancer

In one embodiment, the targeting moiety is attached to a liposome. For example, US 6,245,427 describes a method for targeting a liposome using a protein or peptide. In another example, a cationic lipid component of the liposome is derivatized with a targeting moiety. For example, WO 96/37194 describes converting N-glutaryldioleoylphosphatidyl ethanolamine to a N-hydroxysuccinimide activated ester. The product was then coupled to an RGD peptide.

ROUTE OF DELIVERY

A composition that includes an siRNA can be delivered to a subject by a variety of routes. Exemplary routes include: intravenous, topical, rectal, anal, vaginal, nasal, pulmonary, ocular.

The siRNA molecules of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically include one or more species of siRNA and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include

any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, or intrathecal or intraventricular administration.

The route and site of administration may be chosen to enhance targeting. For example, to target muscle cells, intramuscular injection into the muscles of interest would be a logical choice. Lung cells might be targeted by administering the siRNA in aerosol form. The vascular endothelial cells could be targeted by coating a balloon catheter with the siRNA and mechanically introducing the DNA.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water, syrups, elixirs or non-aqueous media, tablets, capsules, lozenges, or troches. In the case of tablets, carriers that can be used include lactose, sodium citrate and salts of phosphoric acid. Various disintegrants such as starch, and lubricating agents such as magnesium stearate, sodium lauryl sulfate and talc, are commonly used in tablets. For oral administration in capsule form, useful diluents are lactose and high molecular weight polyethylene glycols. When aqueous suspensions are required for oral use, the nucleic acid compositions can be combined

with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring agents can be added.

Compositions for intrathecal or intraventricular administration may include sterile aqueous solutions that may also contain buffers, diluents and other suitable additives.

Formulations for parenteral administration may include sterile aqueous solutions that may also contain buffers, diluents and other suitable additives. Intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir. For intravenous use, the total concentration of solutes should be controlled to render the preparation isotonic.

For ocular administration, ointments or droppable liquids may be delivered by ocular delivery systems known to the art such as applicators or eye droppers. Such compositions can include mucomimetics such as hyaluronic acid, chondroitin sulfate, hydroxypropyl methylcellulose or poly(vinyl alcohol), preservatives such as sorbic acid, EDTA or benzylchronium chloride, and the usual quantities of diluents and/or carriers.

Topical Delivery

In a preferred embodiment, an siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) is delivered to a subject via topical administration. "Topical administration" refers to the delivery to a subject by contacting the formulation directly to a surface of the subject. The most common form of topical delivery is to the skin, but a composition disclosed herein can also be directly applied to other surfaces of the body, *e.g.*, to the eye, a mucous membrane, to surfaces of a body cavity or to an internal surface. As mentioned above, the most common topical delivery is to the skin. The term encompasses several routes of administration including, but not limited to, topical and transdermal. These modes of administration typically include penetration of the skin's permeability barrier and efficient delivery to the target tissue or stratum. Topical administration can be used as a means to penetrate the epidermis and dermis and ultimately achieve systemic delivery of the composition. Topical administration can also be used as a means to selectively

deliver oligonucleotides to the epidermis or dermis of a subject, or to specific strata thereof, or to an underlying tissue.

The term "skin," as used herein, refers to the epidermis and/or dermis of an animal. Mammalian skin consists of two major, distinct layers. The outer layer of the skin is called the epidermis. The epidermis is comprised of the stratum corneum, the stratum granulosum, the stratum spinosum, and the stratum basale, with the stratum corneum being at the surface of the skin and the stratum basale being the deepest portion of the epidermis. The epidermis is between 50 μm and 0.2 mm thick, depending on its location on the body.

Beneath the epidermis is the dermis, which is significantly thicker than the epidermis. The dermis is primarily composed of collagen in the form of fibrous bundles. The collagenous bundles provide support for, inter alia, blood vessels, lymph capillaries, glands, nerve endings and immunologically active cells.

One of the major functions of the skin as an organ is to regulate the entry of substances into the body. The principal permeability barrier of the skin is provided by the stratum corneum, which is formed from many layers of cells in various states of differentiation. The spaces between cells in the stratum corneum is filled with different lipids arranged in lattice-like formations that provide seals to further enhance the skins permeability barrier.

The permeability barrier provided by the skin is such that it is largely impermeable to molecules having molecular weight greater than about 750 Da. For larger molecules to cross the skin's permeability barrier, mechanisms other than normal osmosis must be used.

Several factors determine the permeability of the skin to administered agents. These factors include the characteristics of the treated skin, the characteristics of the delivery agent, interactions between both the drug and delivery agent and the drug and skin, the dosage of the drug applied, the form of treatment, and the post treatment regimen. To selectively target the epidermis and dermis, it is sometimes possible to formulate a composition that comprises one or more penetration enhancers that will enable penetration of the drug to a preselected stratum.

Transdermal delivery is a valuable route for the administration of lipid soluble therapeutics. The dermis is more permeable than the epidermis and therefore

absorption is much more rapid through abraded, burned or denuded skin. Inflammation and other physiologic conditions that increase blood flow to the skin also enhance transdermal adsorption. Absorption via this route may be enhanced by the use of an oily vehicle (inunction) or through the use of one or more penetration enhancers. Other effective ways to deliver a composition disclosed herein via the transdermal route include hydration of the skin and the use of controlled release topical patches. The transdermal route provides a potentially effective means to deliver a composition disclosed herein for systemic and/or local therapy.

In addition, iontophoresis (transfer of ionic solutes through biological membranes under the influence of an electric field) (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 163), phonophoresis or sonophoresis (use of ultrasound to enhance the absorption of various therapeutic agents across biological membranes, notably the skin and the cornea) (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 166), and optimization of vehicle characteristics relative to dose position and retention at the site of administration (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 168) may be useful methods for enhancing the transport of topically applied compositions across skin and mucosal sites.

The compositions and methods provided may also be used to examine the function of various proteins and genes *in vitro* in cultured or preserved dermal tissues and in animals. The invention can be thus applied to examine the function of any gene. The methods of the invention can also be used therapeutically or prophylactically. For example, for the treatment of animals that are known or suspected to suffer from diseases such as psoriasis, lichen planus, toxic epidermal necrolysis, ertythema multiforme, basal cell carcinoma, squamous cell carcinoma, malignant melanoma, Paget's disease, Kaposi's sarcoma, pulmonary fibrosis, Lyme disease and viral, fungal and bacterial infections of the skin.

Pulmonary Delivery

A composition that includes an siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) can be administered to a subject by pulmonary delivery. Pulmonary

delivery compositions can be delivered by inhalation by the patient of a dispersion so that the composition, preferably siRNA, within the dispersion can reach the lung where it can be readily absorbed through the alveolar region directly into blood circulation. Pulmonary delivery can be effective both for systemic delivery and for localized delivery to treat diseases of the lungs.

Pulmonary delivery can be achieved by different approaches, including the use of nebulized, aerosolized, micellular and dry powder-based formulations. Delivery can be achieved with liquid nebulizers, aerosol-based inhalers, and dry powder dispersion devices. Metered-dose devices are preferred. One of the benefits of using an atomizer or inhaler is that the potential for contamination is minimized because the devices are self contained. Dry powder dispersion devices, for example, deliver drugs that may be readily formulated as dry powders. An siRNA composition may be stably stored as lyophilized or spray-dried powders by itself or in combination with suitable powder carriers. The delivery of a composition for inhalation can be mediated by a dosing timing element which can include a timer, a dose counter, time measuring device, or a time indicator which when incorporated into the device enables dose tracking, compliance monitoring, and/or dose triggering to a patient during administration of the aerosol medicament.

The term "powder" means a composition that consists of finely dispersed solid particles that are free flowing and capable of being readily dispersed in an inhalation device and subsequently inhaled by a subject so that the particles reach the lungs to permit penetration into the alveoli. Thus, the powder is said to be "respirable." Preferably the average particle size is less than about 10 μm in diameter preferably with a relatively uniform spheroidal shape distribution. More preferably the diameter is less than about 7.5 μm and most preferably less than about 5.0 μm. Usually the particle size distribution is between about 0.1 μm and about 5 μm in diameter, particularly about 0.3 μm to about 5 μm.

The term "dry" means that the composition has a moisture content below about 10% by weight (% w) water, usually below about 5% w and preferably less it than about 3% w. A dry composition can be such that the particles are readily dispersible in an inhalation device to form an aerosol.

The term "therapeutically effective amount" is the amount present in the composition that is needed to provide the desired level of drug in the subject to be treated to give the anticipated physiological response.

The term "physiologically effective amount" is that amount delivered to a subject to give the desired palliative or curative effect.

The term "pharmaceutically acceptable carrier" means that the carrier can be taken into the lungs with no significant adverse toxicological effects on the lungs.

The types of pharmaceutical excipients that are useful as carrier include stabilizers such as human serum albumin (HSA), bulking agents such as carbohydrates, amino acids and polypeptides; pH adjusters or buffers; salts such as sodium chloride; and the like. These carriers may be in a crystalline or amorphous form or may be a mixture of the two.

Bulking agents that are particularly valuable include compatible carbohydrates, polypeptides, amino acids or combinations thereof. Suitable carbohydrates include monosaccharides such as galactose, D-mannose, sorbose, and the like; disaccharides, such as lactose, trehalose, and the like; cyclodextrins, such as 2-hydroxypropyl-.beta.-cyclodextrin; and polysaccharides, such as raffinose, maltodextrins, dextrans, and the like; alditols, such as mannitol, xylitol, and the like. A preferred group of carbohydrates includes lactose, threhalose, raffinose maltodextrins, and mannitol. Suitable polypeptides include aspartame. Amino acids include alanine and glycine, with glycine being preferred.

Additives, which are minor components of the composition of this invention, may be included for conformational stability during spray drying and for improving dispersibility of the powder. These additives include hydrophobic amino acids such as tryptophan, tyrosine, leucine, phenylalanine, and the like.

Suitable pH adjusters or buffers include organic salts prepared from organic acids and bases, such as sodium citrate, sodium ascorbate, and the like; sodium citrate is preferred.

Pulmonary administration of a micellar siRNA formulation may be achieved through metered dose spray devices with propellants such as tetrafluoroethane, heptafluoroethane, dimethylfluoropropane, tetrafluoropropane, butane, isobutane, dimethyl ether and other non-CFC and CFC propellants.

Oral or Nasal Delivery

Both the oral and nasal membranes offer advantages over other routes of administration. For example, drugs administered through these membranes have a rapid onset of action, provide therapeutic plasma levels, avoid first pass effect of hepatic metabolism, and avoid exposure of the drug to the hostile gastrointestinal (GI) environment. Additional advantages include easy access to the membrane sites so that the drug can be applied, localized and removed easily.

In oral delivery, compositions can be targeted to a surface of the oral cavity, *e.g.*, to sublingual mucosa, which includes the membrane of ventral surface of the tongue and the floor of the mouth, or the buccal mucosa, which constitutes the lining of the cheek. The sublingual mucosa is relatively permeable thus giving rapid absorption and acceptable bioavailability of many drugs. Further, the sublingual mucosa is convenient, acceptable and easily accessible.

The ability of molecules to permeate through the oral mucosa appears to be related to molecular size, lipid solubility and peptide protein ionization. Small molecules, less than 1000 daltons appear to cross mucosa rapidly. As molecular size increases, the permeability decreases rapidly. Lipid soluble compounds are more permeable than non-lipid soluble molecules. Maximum absorption occurs when molecules are un-ionized or neutral in electrical charges. Therefore charged molecules present the biggest challenges to absorption through the oral mucosae.

A pharmaceutical composition of siRNA may also be administered to the buccal cavity of a human being by spraying into the cavity, without inhalation, from a metered dose spray dispenser, a mixed micellar pharmaceutical formulation as described above and a propellant. In one embodiment, the dispenser is first shaken prior to spraying the pharmaceutical formulation and propellant into the buccal cavity.

Devices

SiRNA (or a DNA which encodes it or a precursor, *e.g.*, a larger RNA which can be processed into siRNA) can be disposed on or in a device, *e.g.*, a device that can be implanted or otherwise placed in a subject. Exemplary devices include devices which are introduced into the vasculature, *e.g.*, devices inserted into the lumen of a

vascular tissue, or which devices themselves form a part of the vasculature, including stents, catheters, heart valves, and other vascular devices. These devices, *e.g.*, catheters or stents, can be placed in the vasculature of the lung, heart, or leg.

Other devices include non-vascular devices, *e.g.*, devices implanted in the peritoneum, or in organ or glandular tissue, *e.g.*, artificial organs. The device can release a therapeutic substance in addition to an siRNA, *e.g.*, a device can release insulin.

Other devices include artificial joints, *e.g.*, hip joints, and other orthopedic implants.

In one embodiment, unit doses or measured doses of a composition that includes siRNA are dispensed by an implanted device. The device can include a sensor that monitors a parameter within a subject. For example, the device can include pump, *e.g.*, and, optionally, associated electronics.

Tissue, *e.g.*, cells or organs can be treated with siRNA (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor) ex vivo and then administered or implanted in a subject.

The tissue can be autologous, allogenic, or xenogenic tissue. *E.g.*, tissue can be treated to reduce graft v. host disease. In other embodiments, the tissue is allogenic and the tissue is treated to treat a disorder characterized by unwanted gene expression in that tissue. *E.g.*, tissue, *e.g.*, hematopoietic cells, *e.g.*, bone marrow hematopoietic cells, can be treated to inhibit unwanted cell proliferation.

Introduction of treated tissue, whether autologous or transplant, can be combined with other therapies.

In some implementations, the siRNA treated cells are insulated from other cells, *e.g.*, by a semi-permeable porous barrier that prevents the cells from leaving the implant, but enables molecules from the body to reach the cells and molecules produced by the cells to enter the body. In one embodiment, the porous barrier is formed from alginate.

In one embodiment, a contraceptive device is coated with or contains an siRNA composition (or a precursor, *e.g.*, a larger dsRNA which can be processed into an siRNA, or a DNA which encodes an siRNA or precursor). Exemplary devices include condoms, diaphragms, IUD (implantable uterine devices, sponges, vaginal

sheaths, and birth control devices. In one embodiment, the siRNA is chosen to inactive sperm or egg. In another embodiment, the siRNA is chosen to be complementary to a viral or pathogen RNA, *e.g.*, an RNA of an STD. In some instances, the siRNA composition can include a spermicide.

DOSAGE

The present invention encompasses polynucleotide agents that modulate gene expression or activity. In general, for therapeutics, a patient in need of such therapy is administered a compound in accordance with the invention, commonly in a pharmaceutically acceptable carrier, in doses ranging from, *e.g.*, 0.01 μg to 100 mg per kg of body weight (*e.g.*, less than 5 mg, 2 mg, 1 mg, 100 μg, 50 μg, 10 μg, 5 μg, 1 μg, 0.1 μg, 0.01 μg, or 0.001 μg, and, optionally, at least 0.001 μg, 0.01 μg, 0.1 μg, or 1 μg) per kg of body weight. The dosage can also depend on the age of the subject and the severity of the disease state being treated. Particularly preferred dosages are less than2, 1, or 0.1 mg/kg of body weight.

Further, the treatment regimen may last for a period of time that will vary depending upon the nature of the particular disease, its severity and the overall condition of the patient. In preferred embodiments the dosage may be delivered no more than once per day, *e.g.*, no more than once per 24, 36, 48, or more hours, *e.g.*, no more than once for every 5 or 8 days. Following treatment, the patient can be monitored for changes in his condition and for alleviation of the symptoms of the disease state. The dosage of the compound may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disease state is observed, if the disease state has been ablated, or if undesired side-effects are observed.

The effective dose can be administered in a single dose or in two or more doses, as desired or considered appropriate under the specific circumstances. If desired to facilitate repeated or frequent infusions, implantation of a delivery device, *e.g.*, a pump, semi-permanent stent (*e.g.*, intravenous, intraperitoneal, intracisternal or intracapsular), or reservoir may be advisable.

In some cases, a patient is treated with a siRNA in conjunction with other therapeutic modalities. For example, a patient being treated for a viral disease, *e.g.* an HIV associated disease (*e.g.*, AIDS), may be administered an siRNA specific for a target gene essential to the virus in conjunction with a known antiviral agent (*e.g.*, a protease inhibitor or reverse transcriptase inhibitor). In another example, a patient being treated for cancer may be administered an siRNA specific for a target essential for tumor cell proliferation in conjunction with a chemotherapy.

Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the compound of the invention is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight (see US 6,107,094).

The concentration of the siRNA composition is an amount sufficient to be effective in treating or preventing a disorder or to regulate a physiological condition in humans. The concentration or amount of siRNA administered will depend on the parameters determined for the agent and the method of administration, *e.g.* nasal, buccal, pulmonary. For example, nasal formulations tend to require much lower concentrations of some ingredients in order to avoid irritation or burning of the nasal passages. It is sometimes desirable to dilute an oral formulation up to 10-100 times in order to provide a suitable nasal formulation.

Certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a dsRNA can include a single treatment or, preferably, can include a series of treatments. It will also be appreciated that the effective dosage of a dsRNA such as an siRNA used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein. For example, the subject can be monitored after administering an siRNA composition. Based on information from the monitoring, an additional amount of the siRNA composition can be administered.

Dosing is dependent on severity and responsiveness of the disease condition to be treated, with the course of treatment lasting from several days to several months, or

until a cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual compounds, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. In some embodiments, the animal models include transgenic animals that express a human gene, *e.g.* a gene that produces a target RNA. The transgenic animal can be deficient for the corresponding endogenous RNA. In another embodiment, the composition for testing includes an siRNA that is complementary, at least in an internal region, to a sequence that is conserved between the target RNA in the animal model and the target RNA in a human.

OTHER EMBODIMENTS

In yet another embodiment, dsRNAs are produced in a cell *in vivo*, *e.g.*, from exogenous DNA templates that are delivered into the cell. For example, the DNA templates can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (U.S. Pat. No. 5,328,470), or by stereotactic injection (see, *e.g.*, Chen *et al.* (1994) Proc. Natl. Acad. Sci. USA 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. The DNA templates, for example, can include two transcription units, one that produces a transcript that includes the top strand of a dsRNA and one that produces a transcript that includes the bottom strand of a dsRNA. When the templates are transcribed, the dsRNA is produced, and processed into siRNA fragments that mediate gene silencing.

While this invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

WHAT IS CLAIMED IS:

1. A method of modulating gene expression, comprising administering two different nucleic acids, wherein:

the first nucleic acid is substantially identical to a first region of a target gene; and

the second nucleic acid is substantially identical to a second region of the target gene;

thereby modulating gene expression.

2. The method of claim 1, wherein first and second nucleic acids are RNA.

3. The method of claim 2, wherein DNA encoding the first and second RNAs are administered.

4. The method of claim 1, wherein the first RNA is substantially identical to a noncoding region of the target gene.

5. The method of claim 1, wherein the first RNA is substantially identical to a coding region of the target gene.

6. The method of claim 1, wherein the first RNA is substantially identical to a nontranscribed region of the target gene.

7. The method of claim 1, wherein the first RNA is substantially identical to a transcribed region of the target gene.

8. The method of claim 1, wherein the second RNA is substantially identical to a nontranscribed region of the target gene.

9. The method of claim 1, wherein the second RNA is substantially identical to a transcribed region of the target gene.

10. The method of claim 1, wherein the second RNA is substantially identical to a noncoding region of the target gene.

11. The method of claim 1, wherein the second RNA is substantially identical to a coding region of the target gene.

12. The method of claim 6, wherein the second RNA is substantially identical to a nontranscribed region of the target gene.

13. The method of claim 1, wherein at least 3 different nucleic acids are administered.

14. The method of claim 1, wherein at least 5 different nucleic acids are administered.

15. The method of claim 1, wherein at least 10 different nucleic acids are administered.

16. The method of claim 1, wherein the first and second nucleic acids are not identical in sequence to the target gene.

17. The method of claim 13, wherein the at least 3 different nucleic acids are substantially identical to a nontranscribed region.

18. The method of claim 14, wherein the at least 5 different nucleic acids are substantially identical to a nontranscribed region.

19. The method of claim 15, wherein the at least 10 different nucleic acids are substantially identical to a nontranscribed region.

20. The method of claim 13, wherein the at least 3 different nucleic acids are substantially identical to a noncoding region.

21. The method of claim 14, wherein the at least 5 different nucleic acids are substantially identical to a noncoding region.

22. The method of claim 15, wherein the at least 10 different nucleic acids are substantially identical to a noncoding region.

23. The method of claim 13, wherein the at least 3 different nucleic acids are substantially identical to a transcribed region.

24. The method of claim 14, wherein the at least 5 different nucleic acids are substantially identical to a transcribed region.

25. The method of claim 15, wherein the at least 10 different nucleic acids are substantially identical to a transcribed region.

26. The method of claim 1, wherein the first and second nucleic acids are different.